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Guide to laboratory establishment for plant nutrient analysis

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FAO FERTILIZER AND PLANT NUTRITION BULLETIN

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Preface

This publication provides practical guidelines on establishing service laboratories for the analysis of soil, plants, water and fertilizers (mineral, organic and biofertilizers). A service laboratory needs information on a methodology that is widely acceptable, taking into consideration the ready availability of chemicals, reagents and instruments while ensuring a reasonable degree of accuracy, speed and reproducibility of results. The method needs to be easy to understand for practising technicians who are required to adopt it in a routine manner.

A manual, with simple procedural steps, is considered as providing the best help to the laboratory technicians. This publication provides various analytical methods for estimating soil constituents with the objective of assessing soil fertility and making nutrient recommendations. It describes methods for analysing plant constituents in order to determine the content of various nutrients and the need for their application. For assessing the quality of irrigation water, it presents standard methods for estimating the various parameters and constituents utilized, e.g. electrical conductivity, sodium adsorption ratio, residual sodium carbonate, the ratio of magnesium to calcium, and boron content. In providing the methodology for fertilizer analysis, special consideration has been given to the fact that fertilizers are often statutorily controlled commodities and are traded widely among countries.

This guide also examines biofertilizers. It discusses the bacterial cultures that serve either as a source of nitrogen, such as *Rhizobium*, *Azotobacter* and *Azospirillum*, or for improving the availability of soil phosphorus, such as phosphate-solubilizing microbes. It provides methods for their isolation, identification, multiplication and commercial production. The Reference section includes sources for further detailed information.

This guide details the equipment, chemicals and glassware required in order to establish a composite laboratory with facilities for soil, water and plant analysis. Similarly, it details the requirements for establishing a fertilizer testing laboratory and a biofertilizer testing/production laboratory. To save on the cost of some of the common equipment, facilities and supervision, the analytical facilities required for various materials can be combined. However, it is necessary to ensure that no contamination of the soil by the fertilizers or vice versa takes place. In view of this, even in a composite laboratory (which is otherwise desirable), it is necessary to keep rooms for processing and handling different types of samples separate from one another, while keeping them in close proximity in order to save on time for movement and supervision. The guide takes these considerations into account.

This publication should prove useful to administrators and planners in establishing laboratories, and to technicians through providing detailed and precise procedures for estimations.

List of acronyms, abbreviations and chemical symbols

AAS	Atomic absorption spectrophotometer
Al	Aluminium
AOAC	Association of Official Analytical Chemists, the United States of America
AR	Analytical reagent
As	Arsenic
B	Boron
BAC	Benzalkonium chloride
BGA	Blue-green algae
BOD	Biochemical oxygen demand
C	Carbon
Ca	Calcium
CEC	Cation exchange capacity
Cl	Chlorine
Co	Cobalt
COD	Chemical oxygen demand
CP	Chemically pure
CRYEMA	Congo red yeast extract mannitol agar
Cu	Copper
DAP	Di-ammonium phosphate
DDW	Double-distilled water
DTPA	Diethylenetriamine pentaacetic acid
EBT	Eriochrome Black T
EC	Electrical conductivity
EDTA	Ethylenediamine tetraacetic acid
Eq W	Equivalent weight
Fe	Iron
GLP	Good laboratory practice
H	Hydrogen
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HEPA	High-efficiency particulate air
Hg	Mercury

ICP	Inductively coupled plasma
ICP–AES	Inductively coupled plasma – atomic emission spectroscopy
INM	Integrated nutrient management
IPE	International Plant Analytical Exchange
ISE	International Soil Analytical Exchange
ISO	International Organization for Standardization
K	Potassium
LR	Laboratory reagent
M	Molar
MARSEP	International Manure and Refuse Sample Exchange Programme
mcf	Moisture correction factor
me	Milli-equivalent
mEq W	Milli-equivalent weight
Mg	Magnesium
Mn	Manganese
Mo	Molybdenum
MOP	Muriate of potash
N	Nitrogen
NaOH	Sodium hydroxide
Ni	Nickel
O	Oxygen
OM	Organic matter
P	Phosphorus
PSM	Phosphate-solubilizing micro-organism
RSC	Residual sodium carbonate
S	Sulphur
SAR	Sodium adsorption ratio
Se	Selenium
SETOC	International Sediment Exchange for Tests on Organic Contaminants
SOM	Soil organic matter
SOP	Standard operating procedure
SOP	Sulphate of potash
SSP	Single superphosphate
STPB	Sodium tetraphenyl boron
TEA	Triethanolamine
UV	Ultraviolet
WEPAL	Wageningen Evaluating Programme for Analytical Laboratories
WHC	Water holding capacity
YEMA	Yeast extract mannitol agar
Zn	Zinc

Chapter 1
Introduction

The role of plant nutrients in crop production is well established. There are 16 essential plant nutrients. These are carbon (C), hydrogen (H), oxygen (O), nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), sulphur (S), zinc (Zn), manganese (Mn), copper (Cu), boron (B), molybdenum (Mo) and chlorine (Cl). These nutrient elements have to be available to the crops in quantities as required for a yield target. Any limiting or deficient nutrient (or nutrients) will limit crop growth.

The required nutrients may come from various sources, such as the atmosphere, soil, irrigation water, mineral fertilizers, manures and biofertilizers. The combinations, quantities and integration of nutrients to be supplied from various sources (integrated plant nutrient supply) depend on various factors including the type of crop, soils, availability of various resources, and ultimately on economic considerations, such as the level of production and the costs of inputs and outputs.

Integrated nutrient management (INM) is a well-accepted approach for the sustainable management of soil productivity and increased crop production. To implement this approach successfully, well-equipped testing laboratories, among other things, are needed in order to evaluate the nutrient supplying capacities of various sources.

Accurate and timely analysis helps in determining the requirements of plant nutrients so as to arrange their supply through various sources. The analytical facilities required for chemical analysis of soils, plants, water and fertilizers are broadly identical in nature with a few specific requirements in terms of facilities and chemicals for certain estimations. The facilities for biofertilizer assay are of a highly specialized nature and are different from those required for chemical analysis. In view of this, it is possible to set up integrated facilities for soil, plant, water and fertilizer analysis, and a biofertilizer testing facility can be added (as appropriate) in an adjacent or expanded building. A common facility saves on supervision and other costs, such as common equipment and chemicals.

Depending on the need, different types of laboratories can be set up (Table 1). A soil, plant and water testing laboratory with an annual analysing capacity of about 10 000–12 000 samples requires a building space of about 370 m^2. For a fertilizer testing laboratory with an analysing capacity of 2 000 samples, the space requirement is about 185 m^2. A composite laboratory may require about 480 m^2. Annex 1 provides a laboratory floor plan.

A biofertilizer laboratory with an analysing capacity of 1 000 samples and a production of 25–100 tonnes of biofertilizer per year may require an area of about 270 m^2. Annex 2 provides a floor plan for such a laboratory.

Requirements in terms of equipment, glassware and chemicals have been determined separately for laboratories of types A (Annex 3), B (Annex 4) and D (Annex 5). For the setting up of type C and E laboratories, the requirements in terms of the relevant facilities can be added together. Under budget constraints, there would be advantages in ordering the same chemicals in one order, and in using the same glassware and costly equipment, such as a spectrophotometer and an atomic absorption spectrophotometer (AAS). An experienced chemist would be able to decide on the actual reduction and, thus, achieve cost savings in setting up a composite laboratory.

Some of the methods are common for estimating plant nutrients in soils, plants and fertilizers. Annex 6 summarizes the methods described in this publication.

Given the increasing need for analysis of larger numbers of soil, plant, water and fertilizer samples by the service laboratories to serve the farmers more rapidly and more effectively, various types of equipment/techniques capable of multinutrient analysis may be useful. Annex 7 describes some of them, e.g. autoanalysers and inductively coupled plasma – atomic emission spectroscopy (ICP–AES). However, for service laboratories in developing countries that are limited in terms of facilities, skilled personnel and financial resources, such equipment is not advocated.

TABLE 1
Laboratory types, with analysis capacity

Category	Laboratory type	Type of analysis	Capacity-samples per year
A	Soil, plant & water analysis	Soil	10 000
		Plant	1 000
		Water (irrigation)	500
B	Mineral & organic fertilizer analysis	Mineral fertilizers	1 500
		Organic fertilizers	500
C	Soil, plant, water, mineral & organic fertilizers analysis (A+B)	Soil	10 000
		Plant	1 000
		Water	500
		Mineral fertilizers	1 500
		Organic fertilizers	500
		Biofertilizer	1 000
D	Biofertilizer	Biofertilizer	1 000
E	Soil, plant, water, mineral, organic fertilizer & biofertilizer analysis (C+D)	Soil	10 000
		Plant	1 000
		Water (irrigation)	500
		Mineral fertilizers	1 500
		Organic fertilizers	500

Chapter 2
The basics of an analytical laboratory

In chemical laboratories, the use of acids, alkalis and some hazardous and explosive chemicals is unavoidable. In addition, some chemical reactions during the analysis process may release toxic gases and, if not handled well, may cause an explosion. Inflammable gases are also used as a fuel/heating source. Thus, work safety in a chemical laboratory calls for special care both in terms of the design and construction of the laboratory building, and in the handling and use of chemicals. For chemical operations, it is also necessary to provide special chambers.

The air temperature of the laboratory and work rooms should be maintained constant at 20–25 °C. Humidity should be kept at about 50 percent. Temperature and humidity often affect soil and fertilizer samples. Temperature also affects some chemical operations. Hence, maintaining the temperature and humidity as specified is critical.

Proper air circulation is also important in order to prevent hazardous and toxic fumes and gases from remaining in the laboratory for long. The release of gases and fumes in some specific analytical operations are controlled through fumehoods or trapped in acidic/alkaline solutions and washed through flowing water. The maintaining of a clean and hygienic environment in the laboratory is essential for the good health of the personnel.

Care is required in order to ensure that acids and hazardous chemicals are stored in separate and safe racks. An inventory of all the equipment, chemicals, glassware and miscellaneous items in a laboratory should be maintained (Annex 8 suggests a suitable format). A safe laboratory building should have suitable separate rooms for different purposes and for performing different operations as described below (with a floor plan in Annex 1):

- ➢ Room 1. Reception, sample receipt, and dispatch of reports.
- ➢ Room 2. Sample storage and preparation room (separate for soil/plant and fertilizers).
- ➢ Room 3. Nitrogen digestion/distillation room (with fumehood for digestion).
- ➢ Room 4. Instrument room to house:
  - atomic absorption spectrophotometer (AAS);
  - flame photometer;
  - spectrophotometer;
  - pH meter, conductivity meter;
  - ovens;
  - centrifuge;

- balances;
- water still.

- Room 5. Chemical analysis room (separate for soil/plant and fertilizers):
  - to prepare reagents and chemicals, and to carry out their standardization;
  - to carry out extraction of soil and fertilizer samples with appropriate chemicals/reagents;
  - to carry out titration, colour development, precipitation, filtration, etc.;
  - all other types of chemical work.
- Room 6. Storage room for chemicals and spare equipment.
- Room 7. Office room with computers for data processing and record keeping.

LABORATORY SAFETY MEASURES

Special care is required while operating equipment, handling chemicals and in waste disposal.

Equipment

Electrical cables, plugs and tubing need proper checking in order to avoid accidents. Various types of gas cylinders needed in the laboratory, such as acetylene, nitrous oxide and liquefied petroleum gas, must be kept under watch and properly sealed/capped, and they must be stored in ventilated cupboards.

Chemical reagents

Hazardous chemicals should be stored in plastic bottles. While working with chemicals, such as perchloric acid, a fumehood must be used. Chemicals must be labelled properly, indicating their hazardous nature.

Bottles with inflammable substances need to be stored in stainless-steel containers.

Waste disposal

Each country has special rules and methods for the disposal of hazardous waste.

Cyanides, chromates, arsenic (As), selenium (Se), cobalt (Co) and molybdate are commonly used but hazardous chemicals. They should never be disposed of in the laboratory sink but collected in a metal container for proper disposal at the specified places and in the manner described in national legislation for waste disposal.

General rules and requirements

General safety rules and requirements for personnel working in a laboratory are:

- Learn safety rules and the use of first-aid kits. Keep the first-aid kit handy in a conspicuous place in the laboratory.
- Personal safety aids, such as laboratory coats, protective gloves, safety glasses, face shields and proper footwear, should be used.

- Observe normal laboratory safety practice in connecting equipment to the power supply, in handling chemicals and in preparing solutions of reagents. All electrical work must be done by qualified personnel.
- Maintain an instrument manual and logbook for each item of equipment in order to avoid mishandling, accidents and damage to equipment.
- Keep work tables/spaces clean. Clean up spillage immediately.
- Wash hands after handling toxic/hazardous chemicals.
- Never suck the chemicals by mouth but use automatic pipetting devices.
- Use forceps/tongs to remove containers from hotplates/ovens/furnaces.
- Do not use laboratory glassware for eating/drinking.
- Never open a centrifuge cover until the machine has stopped.
- Add acid to water and not water to acid when diluting the acid.
- Always put labels on bottles, vessels and wash-bottles containing reagents, solutions, samples and water.
- Handle perchloric acid and hazardous chemicals in fumehoods.
- With the wet oxidation method of sample digestion, destroy organic matter (OM) first with nitric acid.
- Read the labels on the bottles before opening them.

LABORATORY QUALITY ASSURANCE/CONTROL

For uniformity of expression and understanding, this guide adopts the definitions of the terms quality, quality assurance and quality control as defined by the International Organization for Standardization (ISO) and also those compiled in FAO Soils Bulletin No. 74 (1998).

Quality

Quality is defined as the total features and characteristics of a product or service that bear on its ability to satisfy a stated and implied need. A product can be stated to possess good quality if it meets the predetermined parameters. In the case of an analytical laboratory, the quality of the laboratory may be considered adequate and acceptable if it has the capacity to deliver the analytical results on a product within the specified limits of errors and as per other agreed conditions of cost and time of analysis so as to enable an acceptable judgement on the product quality.

Quality assurance

As per the ISO, quality assurance means the assembly of all planned and systematic actions necessary to provide adequate confidence that a product, a process or a service will satisfy given quality requirements. Another independent laboratory/ person checks the results of these actions in order to confirm the pronouncement on the quality of a product by a given laboratory.

Quality control

Quality control is an important part of quality assurance, and the ISO defines it as the operational techniques and activities that are used to satisfy quality

requirements. Quality assessment or evaluation is necessary to see whether the activities performed to verify quality are effective. Thus, an effective check on all the activities and processes in a laboratory can only ensure that the results pronounced on a product quality are within the acceptable parameters of accuracy.

In a quality control system, implementing the following steps properly ensures that the results delivered are acceptable and verifiable by another laboratory:

- check on the performance of the instruments;
- calibration or standardization of instruments and chemicals;
- adoption of sample check system as a batch control within the laboratory;
- external check: interlaboratory exchange programme.

To ensure the obtaining of accurate and acceptable results of analysis on a sample, the laboratory has to operate in a well-regulated manner, one where the equipment is properly calibrated and the methods and techniques employed are scientifically sound, which will give reproducible results. For ensuring high standards of quality, it is necessary to follow good laboratory practice (GLP). This can be defined as the organizational process and the conditions under which laboratory studies are planned, performed, monitored, recorded and reported. Thus, GLP expects a laboratory to work according to a system of procedures and protocols, while the procedures are also specified as the standard operating procedure (SOP).

STANDARD OPERATING PROCEDURE

As per FAO (1998), an SOP is a document that describes the regularly recurring operations relevant to the quality of the investigation. The purpose of an SOP is to carry out the operation correctly and always in the same manner. An SOP should be available at the place where the work is done. If, for justifiable reasons, any deviation is allowed from the SOP, the changed procedure should be documented fully.

In a laboratory, an SOP may be prepared for:

- safety precautions;
- procedures for operating instruments;
- analytical methods and preparation of reagents;
- registration of samples.

To sum up, all operations have to be documented properly in order to prevent any ad hoc approach to operations.

ERROR, PRECISION, ACCURACY AND DETECTION LIMIT

Error

Error is an important component of analysis. In any analysis, where the quantity is measured with the greatest exactness that the instrument, method and observer are capable of, it is found that the results of successive determinations differ to a greater or lesser extent. The average value is accepted as most probable. This may not always be the true value. In some cases, the difference in the successive values may be small, in some cases it may be large; the reliability of the result depends on

the magnitude of this difference. There could be a number of factors responsible for this difference, which is also referred to as error. In absolute terms, error is the difference between the observed or measured value and the true or most probable value of the quantity measured. The absolute error is a measure of the accuracy of the measurement. Therefore, the accuracy of a determination may be defined as the concordance between it and the true or most probable value. The relative error is the absolute error divided by the true or most probable value.

The error may be caused by any deviation from the prescribed steps to be taken in analysis. The purity of chemicals, their concentration/strength, the accuracy of the instruments and the skill of the technician are important factors.

Precision and accuracy

In analysis, other important terms are precision and accuracy. Precision is defined as the concordance of a series of measurements of the same quantity. The mean deviation or the relative mean deviation is a measure of precision.

Accuracy expresses the correctness of a measurement, while precision expresses the reproducibility of a measurement. Precision always accompanies accuracy, but a high degree of precision does not imply accuracy. In ensuring high accuracy in analysis, accurate preparation of reagents including their perfect standardization is critical. The purity of chemicals is also important. For all estimations where actual measurement of a constituent of the sample in terms of the "precipitate formation" or formation of "coloured compound" or "concentration in the solvent" is a part of the steps in estimation, chemical reagents involved in such aspects must always be of high purity, which is known as analytical-reagent (AR) grade.

Detection limits

In the analysis for elements, particularly trace elements in soils, plants and fertilizers and for environmental monitoring, the need arises to measure very low contents of analytes. Modern equipment is capable of such estimation. However, while selecting the equipment and the testing method for such a purpose, it is important to have information about the lowest limits to which analytes can be detected or determined with sufficient confidence. Such limits are called detection limits or lower limits of detection.

The capacity of the equipment and the method may be such that it can detect the traces of analyte in the sample. In quantitative terms, the lowest contents of such analytes may be decided through appropriate research as the values of interpretable significance. The service laboratories are generally provided with such limits.

QUALITY CONTROL OF ANALYTICAL PROCEDURES

Independent standards

The ultimate aim of the quality control measures is to ensure the production of analytical data with a minimum of error and with consistency. Once an appropriate

method has been selected, its execution has to be done with utmost care. To check and verify the accuracy of analysis, independent standards are used in the system. The extent of deviation of an analytical value on a standard sample indicates the accuracy of the analysis. Independent standards can be prepared in the laboratory from pure chemicals. When a new standard is prepared, the remainder of the old ones always has to be measured as a mutual check. If the results are not within acceptable levels of accuracy, the process of calibration, preparation of the standard curve and the preparation of reagents should be repeated until acceptable results are obtained on the standard sample. After ensuring this, analysis on an unknown sample can start.

Apart from independent standards, certified reference samples can also be used as "standard". Such samples are obtained from other selected laboratories where the analysis on a prepared standard is carried out by more than one laboratory. Such samples, along with the accompanying analytical values, are used as a check to ensure the accuracy of analysis.

Use of blank

A blank determination is an analysis without the analyte or attribute; in other words, it is an analysis without a sample, going through all steps of the procedure with the reagents only. The use of a blank accounts for any contamination in the chemicals used in actual analysis. The "estimate" of the blank is subtracted from the estimates of the samples. "Sequence control" samples are used in long batches in automated analysis. Generally, two samples, one with a low content and the other with a very high content of known analyte (but the contents falling within the working range of the method) are used as standards to monitor the accuracy of analysis.

Blind sample

A blind sample is one with a known content of analyte. The head of the laboratory inserts this sample in batches and at times unknown to the analyst. Various types of sample material may serve as blind samples, such as control samples or sufficiently large leftovers of test samples (analysed several times). It is essential that the analyst be aware of the possible presence of a blind sample but not be able to recognize the material as such.

Validation of analysis procedures

Validation is the process of determining the performance characteristics of a method/procedure. It is a prerequisite for assessing the suitability of produced analytical data for the intended use. This implies that a method may be valid in one situation but not in another. If a method is very precise and accurate but expensive for adoption, it may be used only when data with that order of precision are needed. The data may be inadequate if the method is less accurate than required. Two types of validation are followed.

Validation of own procedure

In-house validation of methods or procedures by individual user laboratories is a common practice. Many laboratories use their own version of even well-established methods for reasons of efficiency, cost and convenience. Changes in the liquid–solid ratio in extraction procedures for available soil nutrients, shaking time, etc., result in changed values, hence they need validating. Such changes are often introduced in order to take account of local conditions, the cost of analysis, and the required accuracy and efficiency.

Validation of such changes is a part of quality control in the laboratory. It is also a kind of research project, hence not all types of the laboratories may be in a position to modify the standard method. They should follow the given method as accepted and practised by most other laboratories.

Apart from validating methods, laboratories need to employ a system of internal quality control in order to ensure that they are capable of producing reliable analytical data with a minimum of error. This requires continuous monitoring of operations and systematic day-to-day checking of the data produced in order to decide whether these are reliable enough to be released.

Internal quality control requires implementing the following steps:

- ➢ Use a blank and a control (standard) sample of known composition along with the samples under analysis.
- ➢ Round off the analytical values to the second decimal place. The value of the third decimal place should be omitted if it is lower than 5. If it is higher than 5, the value of the second decimal should be raised by 1.

As quality control systems rely heavily on control samples, the sample preparation must be done with great care in order to ensure that:

- ➢ the sample is homogenous;
- ➢ the sample material is stable;
- ➢ the material has uniform and correct particle size as sieved through a standard sieve;
- ➢ relevant information, such as properties of the sample and the concentration of the analyte, is available.

The sample under analysis must also be processed/prepared in such a way that it has a similar particle size and homogeneity to that of the standard (control) sample.

As and when internal checks reveal an error in the analysis, corrective measures should be taken. The error could be one of calculation or typing. Where not, it requires thorough checks on sample identification, standards, chemicals, pipettes, dispensers, glassware, calibration procedure and equipment. The standard may be old or prepared incorrectly. A pipette may indicate the wrong volume. Glassware may not have been cleaned properly. The equipment may be defective, or the sample intake tube may be clogged in the case of a flame photometer or an AAS. The source of error must be detected and the samples analysed again.

Validation of the standard procedure

This refers to the validation of new or existing methods and procedures intended for use in many laboratories, including procedures accepted by national systems or the ISO. It involves an interlaboratory programme of testing the method by a number of selected renowned laboratories according to a protocol issued to all participants. Validation is relevant not only when non-standard procedures are used but also when validated standard procedures are used, and even more so when variants of standard procedures are introduced. The results of validation tests should be recorded in a validation report, from which the suitability of a method for a particular purpose can be deduced.

Interlaboratory sample and data exchange programme

Where an error is suspected in the procedure and the uncertainty cannot be resolved readily, it is not uncommon to have the sample analysed in another laboratory of the same system/organization. The results of the other laboratory may or may not be biased, hence, doubt may persist. It may be necessary for another accredited laboratory to check the sample in order to resolve the problem.

An accredited laboratory should participate in at least one interlaboratory exchange programme. Such programmes exist at local, regional, national and international level. Laboratory exchange programmes exist for method performance studies and laboratory performance studies.

In such exchange programmes, some laboratories or organizations have devised the system where samples of known composition are sent periodically to the participating laboratory without disclosing the results. The participating laboratory analyses the sample by a given method and obtains the results. This provides a possibility for assessing the accuracy of the method being used by a laboratory, and also information about the adoption of the method suggested by the lead laboratory. Some of these programmes are:

- International Plant Analytical Exchange (IPE) Programme;
- International Soil Analytical Exchange (ISE) Programme.

They come under the Wageningen Evaluating Programme for Analytical Laboratories (WEPAL) of Wageningen Agricultural University, the Netherlands. Other programmes run by Wageningen Agricultural University are:

- International Sediment Exchange for Tests on Organic Contaminants (SETOC);
- International Manure and Refuse Sample Exchange Programme (MARSEP).

Another international organization operating a laboratory and method evaluation programme is the Association of Official Analytical Chemists (AOAC) of the United States of America. One of its most popular programmes is for fertilizer quality control laboratories.

Every laboratory benefits if it becomes part of a sample/method checking and evaluation programme. The system of self-checking within the laboratory also has to be followed regularly.

PREPARATION AND STANDARDIZATION OF REAGENT SOLUTIONS

Chemical reagents are manufactured and marketed in different grades of purity. In general, the purest reagents are marketed as "analytical reagent" or AR-grade. Other labels are "LR", meaning laboratory reagent, and "CP", meaning chemically pure (details in Annex 9). The strength of chemicals is expressed as normality or molarity. Therefore, it is useful to have some information about the strength of the acids and alkalis most commonly used in chemical laboratories (Table 2).

Some important terms that are often used in a laboratory for chemical analysis are defined/explained below.

Molarity

A one-molar (M) solution contains one mole or one molecular weight in grams of a substance in each litre of the solution. The molar method of expressing concentration is useful because equal volumes of equimolar solutions contain equal number of molecules.

Normality

The normality of a solution is the number of gram equivalents of the solute per litre of the solution. It is usually designated by the letter N. Semi-normal, penti-normal, desi-normal, centi-normal and milli-normal solutions are often required; these are written as 0.5N, 0.2N, 0.1N, 0.01N and 0.001N, respectively. However, molar expression is preferred because "odd" normalities such as 0.121N are clumsily represented in fractional form.

The definition of normal solution uses the term "equivalent weight". This quantity varies with the type of reaction. Hence, it is difficult to give a clear definition of equivalent weight that covers all reactions. It often happens that the same compound possesses different equivalent weights in different chemical reactions. A situation may arise where a solution has a normal concentration when employed for one purpose and a different normality when used in another chemical reaction. Hence, the system of molarity is preferred.

TABLE 2
Strength of commonly used acids and alkalis

Reagent/ chemical	Normality (approx.)	Molarity (approx.)	Formula weight	% by weight (approx.)	Specific gravity (approx.)	Millilitres required for 1N/litre solution (approx.)	Millilitres required for 1M/litre solution (approx.)
Nitric acid	16.0	16.0	63.0	70	1.42	63.7	63.7
Sulphuric acid	35.0	17.5	98.0	98	1.84	28.0	56.0
Hydrochloric acid	11.6	11.6	36.5	37	1.19	82.6	82.6
Phosphoric acid	45.0	15.0	98.0	85	1.71	22.7	68.1
Perchloric acid	10.5	10.5	100.5	65	1.60	108.7	108.7
Ammonium hydroxide	15.0	15.0	35.0	28	0.90	67.6	67.6

Equivalent weight

The equivalent weight (Eq W) of a substance is the weight in grams that in its reaction corresponds to a gram atom of hydrogen or of hydroxyl, or half a gram atom of oxygen, or a gram atom of univalent ion. When one equivalent weight of a substance is dissolved in one litre, it gives 1N solution. Annex 10 gives the equivalent and molecular weights of some important compounds.

Milli-equivalent weight

Equivalent weight when expressed as milli-equivalent weight (mEq W) means the equivalent weight in grams divided by 1 000. It is commonly expressed as "me". It is the most convenient value because it is the weight of a substance contained in or equivalent to 1 ml of 1N solution. Therefore, it is a unit that is common to both volumes and weights, making it possible to convert the volume of a solution to its equivalent weight, and the weight of a substance to its equivalent volume of solution: number of mEq = volume × normality.

Buffer solutions

Solutions containing either a weak acid and its salt or a weak base and its salt (e.g. $CH_3COOH + CH_3COONa$ and $NH_4OH + NH_4Cl$) possess the characteristic property of resisting changes in pH when some acid or base is added to them. Such solutions are called buffer solutions. The important properties of a buffer solution are:

- It has a definite pH value.
- Its pH value does not alter on keeping for a long time.
- Its pH value is only altered slightly when a strong base or strong acid is added.

Because of the above property, readily prepared buffer solutions of known pH are used in order to check the accuracy of pH meters used in the laboratory.

Titrations

Titration is a process of determining the volume of a substance required to just complete the reaction with a known amount of other substance. The solution of known strength used in the titration is called the titrant. The substance to be determined in the solution is called the titrate.

The completion of the reaction is assessed with the help of an appropriate indicator.

Indicators

A substance that indicates the end point on completion of the reaction is called an indicator. The most commonly used indicators in volumetric analysis are:

- internal indicators,
- external indicators,
- self-indicators.

Internal indicators

Indicators such as methyl red, methyl orange, phenolphthalein and diphenylamine that are added to the solution where reaction occurs are called internal indicators. On completion of the reaction of titrant on titrate, a colour change takes place owing to the presence of the indicator, which also helps in knowing that the titration is complete. The internal indicators used in acid–alkali neutralization solutions are methyl orange, phenolphthalein and bromothymol blue.

The indicator used in precipitation reactions such as titration of neutral solution of NaCl (or chlorine ion) with silver nitrate ($AgNO_3$) solution is $K_2CrO_4^-$. On completion of the titration reaction of $AgNO_3$ with Cl, when no more Cl is available for reaction with silver ions to form AgCl, the chromium ions combine with Ag^{2+} ions to form sparingly soluble Ag_2CrO_4, which is brick red in colour. This indicates that Cl has been titrated completely and that end point has occurred.

Redox indicators are also commonly used. These are substances that possess different colours in the oxidized and reduced forms. Diphenylamine has a blue-violet colour in the oxidation state and is colourless in reduced condition. Ferrocin gives a blue colour in the oxidation state and a red colour in reduced condition.

External indicators

Some indicators are used outside the titration mixture. Potassium ferricyanide is used as an external indicator in the titration of potassium dichromate and ferrous sulphate in acid medium. In this titration, a few drops of indicator are placed on a white porcelain tile. A glass rod dipped in the solution being titrated is taken out and brought into contact with the drops of indicator on the tile. First, a deep blue colour is noticed, which turns greenish on completion of titration.

Self-indicators

After completion of the reaction, the titrant leaves its own colour owing to its slight excess in minute quantities. In $KMnO_4$ titration with ferrous sulphate, the addition of $KMnO_4$ starts reacting with $FeSO_4$, which is colourless. On completion of titration, the slight excess presence of $KMnO_4$ gives a pink colour to the solution, which acts as a self-indicator and points to the completion of the titration.

Standard solutions

A solution of accurately known strength (or concentration) is called a standard solution. It contains a definite number of gram equivalent or gram mole per litre of solution. If it contains 1 g equivalent weight of a substance/compound, it is 1N solution. If it contains 2 g equivalent weights of the compound, it is 2N.

All titrimetric methods depend on standard solutions that contain known amounts of the reagents in unit volume of the solution. A solution is prepared, having about the desired concentration. This solution is then standardized by

titriting it with another substance that can be obtained in highly purified form. Thus, potassium permanganate solution can be standardized against sodium oxalate, which can be obtained in a high degree of purity as it is easily dried and is non-hygroscopic. Such a substance, whose weight and purity is stable, is called a "primary standard". A primary standard must have the following characteristics:

- It must be obtainable in a pure form or in a state of known purity.
- It must react in one way only under the condition of titration and there must be no side-reactions.
- It must be non-hygroscopic. Salt hydrates are generally not suitable as primary standards.
- Normally, it should have a large equivalent weight in order to reduce the error in weighing.
- An acid or a base should preferably be strong, that is, it should have a high dissociation constant for being used as standards.

A primary standard solution is one that can be prepared directly by weighing the material and with which other solutions of approximate strength can be titrated and standardized. Some primary standards are:

- acids:
  - potassium hydrogen phthalate,
  - benjoic acid;
- bases:
  - sodium carbonate,
  - borax;
- oxidizing agents:
  - potassium dichromate,
  - potassium bromate;
- reducing agents:
  - sodium oxalate,
  - potassium ferrocyanide;
- others:
  - sodium chloride,
  - potassium chloride.

Secondary standard solutions are those that are prepared by dissolving a little more than the gram equivalent weight of the substance per litre of the solution, and then their exact standardization is done with primary standard solution. Some secondary standards are:

- acids:
  - sulphuric acid,
  - hydrochloric acid;
- base:
  - sodium hydroxide.

Standard solutions of all the reagents required in a laboratory must be prepared and kept ready before commencing any analysis. However, their strength should be checked periodically, or fresh reagents should be prepared before analysis.

All titrations involving acidimetry and alkalimetry require standard solutions. These may be prepared either from standard substances by direct weighing, or by standardizing a solution of approximate normality of materials by titrating against a prepared standard. The methods for preparing standard solutions of some non-primary standard substances in common use are given below.

Standardization of hydrochloric acid

Concentrated hydrochloric acid (HCl) is about 11N. Therefore, to prepare a standard solution, say, decinormal (0.1N) of the acid, it is diluted about 100 times. Take 10 ml of acid and make about 1 litre by dilution with distilled water. Titrate this acid against 0.1N Na_2CO_3 (primary standard) using methyl orange as indicator. The colour changes from pink to yellow when the acid is neutralized. Suppose 10 ml of acid and 12 ml of Na_2CO_3 are consumed in the titration, then:

Acid		Alkali
$V_1 \times N_1$	=	$V_2 \times N_2$
$10 \times N_1$	=	12×0.1
$10\ N_1$	=	1.2
N_1	=	0.12

Thus, the normality of the acid is 0.12.

Standardization of sulphuric acid

Similar to above, the normality of sulphuric acid (H_2SO_4) can be determined. It needs to be diluted about 350 times in order to reach about 0.1N because it has a normality of about 35. Then, titrate against standard Na_2CO_3 to determine the exact normality of H_2SO_4.

Standardization of sodium hydroxide

As per the above method, the normality of HCl/H_2SO_4 has been fixed. Therefore, to determine the normality of sodium hydroxide (NaOH), titration is carried out by using either of these standard acids. To determine the molarity, molar standard solutions are used.

In the case of the standardization of NaOH or any other alkali, potassium hydrogen phthalate can also be used as a primary standard instead of titration with secondary standards.

Chapter 3
Soil analysis

Soil is the main source of nutrients for crops. Soil also provides support for plant growth in various ways. Knowledge about soil health and its maintenance is critical to sustaining crop productivity. The health of soils can be assessed by the quality and stand of the crops grown on them. However, this is a general assessment made by the farmers. A scientific assessment is possible through detailed physical, chemical and biological analysis of the soils.

Essential plant nutrients such as N, P, K, Ca, Mg and S are called macronutrients, while Fe, Zn, Cu, Mo, Mn, B and Cl are called micronutrients. It is necessary to assess the capacity of a soil to supply nutrients in order to supply the remaining amounts of needed plant nutrients (total crop requirement - soil supply). Thus, soil testing laboratories are considered nerve centres for nutrient management and crop production systems.

AVAILABLE NUTRIENT CONTENT OF SOILS

Soils may have large amounts of nutrient reserves in them. All or a part of these reserves may not be of any use to crops because they may not be in plant-available form. For the purpose of estimation or analysis of plant-available soil nutrients, such methods are to be used that have been tested/verified for the correlation of nutrients extracted and their plant availability. This guide describes internationally accepted and widely used methods.

Apart from nutrients, soil pH estimation is also critical in the assessment of soil health. Generally, plants prefer soils that are close to either side of neutrality. However, there are acid-loving crops and also crops that can withstand high soil alkalinity. Hence, good crop yields are possible in acid and alkali soils. With proper amendments, still higher yields can be obtained in acid and alkali soils. Soil pH also has a considerable influence on the activity of soil microflora and on the availability of soil nutrients to crops. It is also important to estimate physical properties such as soil texture and soil structure.

SOIL SAMPLING

The methods and procedures for obtaining soil samples vary according to the purpose of the sampling. Analysis of soil samples may be needed for engineering and agricultural purposes. This guide describes soil sampling for agricultural purposes, i.e. for soil fertility evaluation and fertilizer recommendations for crops.

The results of even very carefully conducted soil analyses can only be as good as the soil samples themselves. Thus, the efficiency of a soil testing service depends

on the care and skill with which soil samples are collected. Non-representative samples constitute the largest single source of error in a soil fertility programme. The most important phase of soil analysis takes place not in the laboratory but in the field where the soil is sampled.

Soils vary from place to place. In view of this, efforts should be made to take the samples in such a way that they are fully representative of the field. Only 1–10 g of soil is used for each chemical determination and this sample needs to represent as accurately as possible the entire surface 0–22 cm of soil, weighing about 2 million kg/ha.

Sampling tools and accessories

Depending on the purpose and precision required, the following tools may be needed for taking soil samples:

- a soil auger – it may be a tube, post-hole or screw-type auger or even a spade for taking sample;
- a clean bucket or a tray or a clean cloth – for mixing the soil and subsampling;
- cloth bags of a specific size;
- a copying pencil for markings, and tags for tying cloth bags;
- soil sample information sheet.

Selection of a sampling unit

A visual survey of the field should precede the actual sampling. Note the variation in slope, colour, texture, management and cropping pattern by traversing the field. Demarcate the field into uniform portions, each of which must be sampled separately. Where all these conditions are similar, one field can be treated as a single sampling unit. Such a unit should not exceed 1–2 ha, and it must be an area to which a farmer is willing to give separate attention. The sampling unit is a compromise between expenditure, labour and time on the one hand, and precision on the other.

Sampling procedure

Prepare a map of the area to be covered in a survey showing different sampling unit boundaries. Enter a plan of the number of samples and manner of composite sampling on the map, designating different fields by letters (A, B, C, etc.). Traverse each area separately. Cut a slice of the plough layer at intervals of 15–20 steps or according to the area to be covered. Generally, depending on the size of the field, 10–20 spots must be taken for one composite sample.

Scrape away surface litter to obtain a uniformly thick slice of soil from the surface to the plough depth from each spot. Make a V-shaped cut with a spade to remove a 1–2-cm slice of soil. Collect the sample on the blade of the spade and put it in a clean bucket. In this way, collect samples from all the spots marked for one sampling unit. In the case of hard soil, take samples with the help of an auger from the plough depth and collect them in the bucket.

Pour the soil from the bucket onto a piece of clean paper or cloth, and mix it thoroughly. Spread the soil evenly and divide it into quarters. Reject two opposite quarters and mix the rest of the soil again. Repeat the process until left with about 0.5 kg of the soil. Collect it and put in a clean cloth bag. Mark each bag clearly in order to identify the sample.

The bag used for sampling must always be clean and free from any contamination. If the same bag is to be used a second time, turn it inside out and remove the soil particles. Write the details of the sample on the information sheet (Annex 11 provides a suggested format). Put a copy of this information sheet in the bag. Tie the mouth of the bag carefully.

Precautions

When sampling a soil, bear in mind the following:

- Do not sample unusual areas, such as unevenly fertilized areas, marshy areas, old paths, old channels, old bunds, areas near trees, sites of previous compost piles, and other unrepresentative sites.
- For a soft and moist soil, the tube auger or spade is considered satisfactory. For harder soil, a screw auger may be more convenient.
- Where crops have been planted in rows, collect samples from the middle of the rows in order to avoid the area where fertilizer has been band placed.
- Avoid any type of contamination at all stages. Soil samples should never be stored with fertilizer materials and detergents. Contamination is likely when the soil samples are spread out to dry in the vicinity of stored fertilizers or on floor where fertilizers were stored previously.
- Before putting soil samples in bags, they should be examined for cleanliness as well as for strength.
- The information sheet should be filled in clearly with a copying pencil.

Sampling salt-affected soils

Salt-affected soils may be sampled in two ways. Surface samples should be taken in the same way as for soil fertility analysis. These samples are used to determine the gypsum requirement of the soil. For reclamation purpose, it is necessary to know also the characteristics of lower soil depth. Therefore, such soils are sampled down to a depth of 1 m. The samples may be removed from one to two spots per 0.4 ha where the soil is uniformly salt-affected. Where patches are conspicuous, then all large patches should be sampled separately. Soil is sampled separately for soil depths (about 0.5 kg from each depth) of 0–15, 15–30, 30–60 and 60–100 cm. If a stony layer is encountered during sampling, such a layer should be sampled separately and its depth noted. This is very important and must not be ignored.

Soil samples can be removed by a spade, or if the auger is used, then care should be taken to note the depth of “concretion” (stones) or other impermeable layer (hardpan). If the soil shows evidence of profile development or distinct stratification, samples should be taken by horizon. If a pit is dug and horizons are absent, then mark the vertical side of the pit at depths of 15, 30, 60 and 100 cm from

the surface and collect about 0.5 kg soil from each layer, cutting uniform slices of soil separately. In addition to the above sampling, one surface soil sample should be taken as in the case of normal soil sampling for fertilizer recommendation.

Pack the samples and label the bags in the same way as for normal soil sampling, giving additional information about the depth of the sample. The sheet accompanying the sample must include information on:

- nature of the soil;
- hardness and permeability of the soil;
- cause and source of salinity (where known);
- relief;
- seasonal rainfall;
- irrigation and frequency of waterlogging;
- water table;
- soil management history;
- crop species and conditions of plant cover;
- depth of the hardpan or concretion.

As the salt concentration may vary greatly with vertical or horizontal distance and with moisture and time, it is necessary to keep an account of the time of irrigation and of the amount of irrigation or rain received prior to sampling.

DISPATCH OF SOIL SAMPLES TO THE LABORATORY

Before sending soil samples to the testing laboratory, it is necessary to ensure that proper identification marks are present on the sample bags and labels placed in the bags. It is essential to use a copying pencil and not ink because ink can smudge and become illegible. The best system is to obtain soil sampling bags from the soil testing laboratory with most of the information printed or stencilled on them in indelible ink.

Compare the number and details on the bag with the dispatch list. The serial numbers of different places should be distinguished by putting the identification mark specific for each centre. This may be in letters, e.g. one for the district, another for the block/county, and a third for the village.

Pack the samples properly. Wooden boxes are most suitable for long transport. Sample bags should be packed only in clean bags never used for fertilizer or detergent packing.

Farmers may bring soil samples directly to the laboratory. However, most samples are sent to the laboratories through field extension staff. An organized assembly–processing–dispatch system is required in order to ensure prompt delivery of samples to the laboratory.

PREPARATION OF SOIL SAMPLES FOR ANALYSIS

Handling in the laboratory

As soon as the samples arrive at the soil testing laboratory, they should be checked against the accompanying information list. If the laboratory personnel have

collected the samples themselves, then adequate field notes should have been kept. All unidentifiable samples should be discarded. Information regarding samples should be recorded in a register, and each sample should be given a laboratory number, in addition to the sample number, to help to distinguish it where more than one source of samples is involved.

Drying of samples

Samples received in the laboratory may be moist. They should be dried in wooden or enamelled trays. Care should be taken to maintain the identity of each sample at all stages of preparation. During drying, the trays can be numbered or a plastic tag could be attached. The samples are allowed to dry in the air. Alternatively, the trays may be placed in racks in a hot-air cabinet, whose temperature should not exceed 35 °C and whose relative humidity should be 30–60 percent. Oven drying a soil can cause profound changes in the sample. This step is not recommended as a preparatory procedure despite its convenience. Drying has a negligible effect on total N content, but the nitrate content in the soil changes with time and temperature. Drying at a high temperature affects the microbial population. With excessive drying, soil K may be released or fixed depending on the original level of exchangeable K. Exchangeable K will increase if its original level was less than 1 me/100 g soil (1 cmol/kg) and vice versa, but the effect depends on the nature of clay minerals in the soil. In general, excessive drying, such as oven drying of the soil, affects the availability of most of the nutrients present in the sample and should be avoided. Only air drying is recommended.

Nitrate, nitrite and ammonium determinations must be carried out on samples brought straight from the field. These samples should not be dried. However, the results are expressed on an oven-dry basis by estimating separately the moisture content in the samples.

Post-drying care

After drying, the samples are taken to the preparation room. Air-dried samples are ground with a wooden pestle and mortar so that the soil aggregate is crushed but the soil particles do not break down. Samples of heavy clay soils may have to be ground with an end-runner grinding mill fitted with a pestle of hard wood and rubber lining to the mortar. Pebbles, concretions and stones should not be broken during grinding.

After grinding, the soil is screened through a 2-mm sieve. The practice of passing only a portion of the ground sample through the sieve and discarding the remainder is erroneous. This introduces a positive bias in the sample as the rejected part may include soil elements with differential fertility. Therefore, the entire sample should be passed through the sieve except for concretions and pebbles of more than 2 mm. The coarse portion on the sieve should be returned to the mortar for further grinding. Repeat sieving and grinding until all aggregate particles are fine enough to pass the sieve and only pebbles, organic residues and concretions remain.

If the soil is to be analysed for trace elements, containers made of copper, zinc and brass must be avoided during grinding and handling. Sieves of different sizes can be obtained in stainless steel. Aluminium or plastic sieves are useful alternative for general purposes.

After the sample has passed through the sieve, it must be mixed again thoroughly.

The soil samples should be stored in cardboard boxes in wooden drawers. These boxes should be numbered and arranged in rows in the wooden drawers, which are in turn fitted in a cabinet in the soil sample room.

ANALYTICAL METHODS

The following estimations are generally carried out in a service-oriented soil testing laboratory:

- soil texture,
- soil structure,
- cation exchange capacity (CEC),
- soil moisture,
- water holding capacity,
- pH,
- lime requirement,
- electrical conductivity,
- gypsum requirement,
- organic C,
- total N,
- mineralizable N,
- inorganic N,
- available P,
- available K,
- available S,
- calcium,
- calcium plus magnesium,
- micronutrients – available Zn, Cu, Fe, Mn, B and Mo.

Soil texture

Soil texture (or particle size distribution) is a stable soil characteristic that influences the physical and chemical properties of the soil. The sizes of the soil particles have a direct relationship with the surface area of the particles. Soil particles remain aggregated owing to various types of binding forces and factors. These include the content of OM, other colloidal substances present in the soil, oxides of Fe and aluminium (Al), and the hydration of clay particles. To estimate the content of various sizes of soil particles, the soil sample has to be brought into a dispersed state by removing the various types of binding forces.

In the dispersed soil samples, the soil particles settle down at a differential settling rate according to their size. In the estimation of soil texture, particles of less than 2 mm in diameter are determined separately and characterized as: coarse sand (2.0–0.2 mm); fine sand (0.2–0.02 mm); silt (0.02–0.002 mm); and clay (< 0.002 mm).

The soil sample is dispersed by removing the binding force in soil particles. The settling rate of dispersed particles in water is measured. Large particles are known to settle out of suspension more rapidly than do small particles. This is because larger particles have less specific area and, hence, less buoyancy than smaller particles. Stokes' law (1851) is used to express the relationship. It stipulates that the resistance offered by the liquid to the fall of the particle varies with the radius of the sphere and not with the surface. Accordingly, the formula is:

$$V = \frac{2}{9}\left(\frac{dp - d}{\eta}\right) gr^2$$

where:

- V is the velocity of the fall in centimetres per second;
- g is the acceleration due to gravity;
- dp is the density of the particle;
- d is the density of the liquid;
- r is the radius of the particle in centimetres,
- η is the absolute viscosity of the liquid.

The velocity of fall of the particles with the same density in a given liquid increases with the square of the radius.

With the above principle in view, the particle size distribution is estimated by measuring the amount of different sizes of soil particles present at different calibrated depths in the cylinder containing the suspended soil sample.

The two methods in general use for estimating particle size or soil texture are:

- the international pipette method;
- the Bouyoucos hydrometer method.

Hydrometer method

The hydrometer method is in more common use because it is less time-consuming and easier to follow in a service laboratory. Dispersion is obtained using sodium hexametaphosphate. It requires the following apparatus:

- a balance;
- cylinders – 1 litre and 1.5 litres;
- a glass beaker – 1 litre;
- a metal stirrer with a speed of 1 500 rpm;
- a Bouyoucos hydrometer;
- an oven;
- a thermometer (degrees Celsius).

The reagent is a sodium hexametaphosphate solution containing 50 g of salt per litre of water.

The procedure is:

1. Weigh 50 g of oven-dried, fine-textured soil (100 g for coarse-textured soil) into a baffled stirring cup. Half fill the cup with distilled water and add 10 ml of sodium hexametaphosphate solution.
2. Place the cup on the stirrer and stir until the soil aggregates are broken down. This usually takes 3–4 minutes for coarse-textured soils and 7–8 minutes for fine-textured clay.
3. Quantitatively transfer the stirred mixture to the settling cylinder by washing the cup with distilled water. Fill the cylinder to the lower mark with distilled water after placing the hydrometer in the liquid. Where 100 g of coarse-textured sample was used, fill to the upper mark on the settling cylinder.
4. Remove the hydrometer and shake the suspension vigorously in a back-and-forth manner. Avoid creating circular currents in the liquid as they influence the settling rate.
5. Place the cylinder on a table and record the time. After 20 seconds, carefully insert the hydrometer and read the hydrometer at the end of 40 seconds.
6. Repeat steps 4 and 5 to obtain hydrometer readings within 0.5-g differences of each other. The hydrometer is calibrated to read grams of soil material in suspension.
7. Record the hydrometer readings on the data sheet (Table 3).
8. Measure the temperature of the suspension. For each degree above 20 °C, add 0.36 to the hydrometer reading; for each degree below 20 °C, subtract 0.36 from the hydrometer reading. This is the corrected hydrometer reading.
9. Re-shake the suspension and place the cylinder on a table where it will not be disturbed. Take a hydrometer reading exactly 2 hours later. Correct for temperature as described above.
10. From the percentage of sand, silt and clay calculated on the data sheet, use the textural triangle diagram (Figure 1) to determine the texture class of the soil.

Soil structure

Soil structure is defined as the arrangement of the soil particles. With regard to structure, soil particles refer not only to sand, silt and clay but also to the aggregate or structural elements that have been formed by the aggregation of smaller mechanical fractions. Therefore, the word “particle” refers to any unit that is part of the make-up of the soil, whether a primary unit (sand, silt or clay fraction) or a secondary (aggregate) particle.

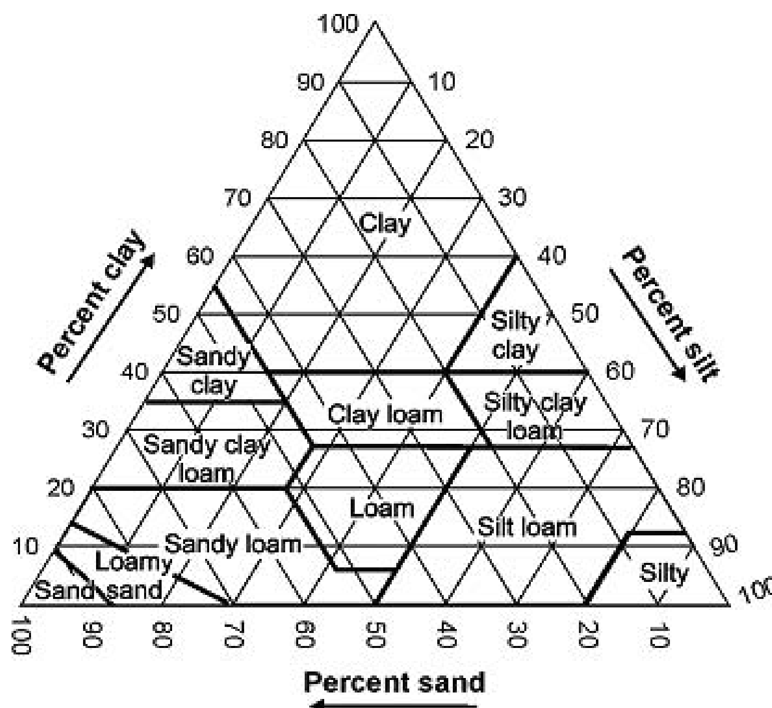
The size, shape and character of the soil structure varies (e.g. cube-like, prism-like or platter-like). On the basis of size, the soil structure is classified as:

- very coarse: > 10 mm;
- coarse: 5–10 mm;
- medium: 2–5 mm;

TABLE 3
Data sheet for recording hydrometer readings

1	Soil sample identification number
2	Soil weight (g)
3	40-second hydrometer reading (g)
4	Temperature of suspension (°C)
5	Corrected 40-second hydrometer reading (g)
6	2-hour hydrometer reading (g)
7	Temperature of suspension (°C)
8	Corrected 2-hour hydrometer reading (g)
9	Grams of sand (the sand settles to the bottom of the cylinder within 40 seconds, therefore, the 40-second corrected hydrometer reading actually gives the grams of silt and clay in suspension. The weight of sand in the sample is obtained by subtracting line 5 from line 2).
10	Grams of clay (the corrected hydrometer reading at the end of 2 hours represents grams of clay in the suspension as all sand and silt has already settled by this time).
11	Percent sand (line 9 ÷ line 2) × 100
12	Percent clay (line 10 ÷ line 2) × 100
13	Percent silt (find the silt by difference. Subtract the sum of the percent sand and clay from 100).
14	Soil class (as per Figure 1)

FIGURE 1
Soil texture classes according to proportions of sand, silt and clay



- fine: 1–2 mm;
- very fine: < 1 mm.

Depending on the stability of the aggregate and the ease of separation, the structure is characterized as:

- poorly developed;
- weakly developed;
- moderately developed;
- well developed;
- highly developed.

The soil structure or aggregate consists of an intermediate grouping of a number of primary particles into a secondary unit. The important factors that facilitate the aggregation of soil particles are:

- clay particles and types of clay minerals;
- cations such as Ca;
- OM;
- colloidal matter such as oxides of Fe and Al;
- plant roots;
- soil microbes and their types (fungi being most effective).

Soil structure influences the extent of pore space in the soil, water holding capacity (WHC), aeration, root movement and nutrient availability. The better and more stable soil aggregates are considered a desirable soil property with regard to plant growth. Therefore, the determination of soil structure is an important exercise in a soil fertility evaluation programme. An aggregate analysis aims to measure the percentage of water-stable secondary particles in the soil and the extent to which the finer mechanical separates are aggregated into coarser fractions.

The determination of aggregate or clod-size distribution involves procedures that depend on the disintegration of soil into clods and aggregates. The resulting aggregate-size distribution depends on the manner and condition in which the disintegration is brought about. For the measurements to have practical significance, the disruptive forces causing disintegration should compare closely with the forces expected in the field. The field condition, particularly with respect to soil moisture, should be compared with the moisture condition adopted for soil disintegration in the laboratory. The sampling of soil and the subsequent disintegration of clods in regard to seed bed preparation for upland crops should be carried out under air-dry conditions for dry-sieve analysis. A rotary sieve shaker is ideal for dry sieving. Similarly, the processes of wetting, disruption of dry aggregates, and screening of aggregates should be compared with the disruptive actions of water and mechanical forces of tillage under wetland conditions. Vacuum wetting of dry soil largely simulates the process of wetting *in situ*, particularly in the subsurface layers. However, the surface soil clods experience large-scale disruption when they are immersed in water at atmospheric pressure. The reproducibility of the size distribution of clods should be the criterion for

deciding the method of wetting by either vacuum wetting or immersion in water. Immersion wetting is closer to wetting of surface soil by irrigation.

After wetting, aggregates of different sizes can be obtained through several methods, e.g. sedimentation, elutriation and sieving. However, sieving under water compares more closely with the disruptive actions of water and other mechanical forces as experienced during wetland rice field preparation.

Dry aggregate analysis

The size distribution of dry clods is measured by dry sieving analysis performed on an air-dried bulk soil sample, either manually or with the help of a rotary sieve shaker (Gupta and Ghil Dyal, 1998).

The apparatus required consists of:

- a nest of sieves, 20 cm in diameter and 5 cm in height, with screens having 25.0, 10.0, 5.0, 2.0, 1.0, 0.5 and 0.25-mm-diameter round openings, with a pan and a lid;
- a rotary sieve shaker;
- some aluminium cans;
- a balance;
- a spade;
- a brush;
- some polyethylene bags;
- some labels.

Under this procedure, collect a bulk soil sample from the tilled field with the help of a 20-cm-diameter and 10-cm-height ring. Place the ring on the tilled soil and press until level with the surface. Remove the loose soil within the ring and collect it in a polyethylene bag.

Put one label indicating the depth and soil profile inside the bag, and tie another label with the bag. Take the soil sample to the laboratory and air-dry it.

Spread the soil on a sheet of paper and prepare the subsamples by "quartering". Cone the mixed soil material in the centre of the mixing sheet with care to make it symmetrical with respect to fine and coarse soil material. Flatten the cone and divide it through the centre with a flat metal spatula or metal sheet, moving one-half to one side. Further divide each half into halves; separating the four portions into separate piles or "quarters". Weigh the subsamples from two of these quarters and use them for clod-size and aggregate distribution analysis as duplicates. Transfer the weighed soil sample to the top sieve of the nest of sieves with the 5.0, 2.0, 1.0, 0.5 and 0.25-mm-diameter round openings and a pan at the bottom. Cover the top sieve with the lid, and place the nest of sieves on a rotary shaker. Switch on the shaker for 10 minutes, and then remove the sieves. Collect the soil retained on each screen in the pre-weighed aluminium cans (with the help of a small brush), and weigh the cans with the soil.

If the percentage of dry aggregates on the 5-mm sieve exceeds 25 percent, transfer these aggregates to a nest of sieves with 25.0, 10.0 and 5.0-mm sieves along with a pan. Cover the top sieve containing the aggregates with a lid, and place

the nest of sieves on the rotary sieve shaker. Switch on the motor for 10 minutes, and proceed as above for the estimation of aggregate-size distribution. Analyse the duplicate sample following the same procedure, and calculate the percentage distribution of dry aggregates retained on each sieve.

Dry the duplicate 100-g sample in an oven for 24 hours at 105 °C to calculate the oven-dry weight of the soil sample. The steps in the calculation are:

- Weight of aggregates in each sieve group = (Wt. of aggregates + Can) – Wt. of can
- Percent distribution of aggregates in each size group:

$$\frac{\text{Weight of Aggregates in each size group}}{\text{Total weight of soil}} \times 100$$

- Oven-dry weight:

$$\text{Oven-dry wt. of Aggregate (\%)} = \frac{\text{Air-dry wt.(\%)} \times 100}{100 + \text{Moisture \%}}$$

Wet aggregate analysis

For wet aggregate analysis (Gupta and Ghil Dyal, 1998), the apparatus required consists of:

- a mechanical oscillator powered by a gear-reduction motor with an amplitude of oscillation of 3.8 cm and a frequency of 30–35 cycles per minute;
- two sets of sieves, each 20 cm in diameter and 5 cm high, with screen openings of 5.0, 2.0, 1.0, 0.5, 0.25 and 0.1 mm in diameter,
- two Büchner funnels, 15 cm in diameter, with rubber stoppers;
- two vacuum flasks of 1-litre capacity;
- a suction pump or aspirator;
- a rubber policeman;
- twelve aluminium cans;
- some perforated cans;
- a sand bath;
- some filter papers.

The reagents required are:

- 5-percent sodium hexametaphosphate;
- 4-percent sodium hydroxide.

Among the different procedures adopted, wetting the samples under vacuum is suggested because the rate of wetting influences the slaking of crumbs. The time of sieving ranges from 10 minutes to 30 minutes depending on the type of wetting. Baver and Rhodes (1932) suggest 10 minutes pre-shaking of the soil sample in a reciprocating shaker or end-to-end shaker for fine-textured soil.

The technique used by Yoder (1936) and subsequently improved by the Soil Science Society of America's Committee on Physical Analysis is generally used for determining the size distribution of water-stable aggregates. Take the soil sample when it is moist and friable. By applying mild stress, break it into smaller

aggregates that can pass through an 8-mm screen. Put the sieved soil sample on a watch glass for wetting by either vacuum soaking or the immersion method. For vacuum wetting, place the sample in a vacuum desiccator containing de-aerated water at the bottom. Evacuate the desiccator until the pressure inside drops to about 3 mm and water starts boiling. Now, allow water to enter through the top of the desiccator and to flow into the watch glass holding the sample. Add enough water to cover the soil sample. Then, take the soil sample out of the desiccator.

Prepare four soil samples of 25 g each. Place a set of duplicate samples in an oven in order to determine the moisture content. Transfer another set of saturated duplicate soil samples to the top sieve of the nest of sieves (5.0, 2.0, 1.0, 0.5, 0.25 and 0.1 mm), and spread with the help of a glass rod and a slow jet of water. Remove the bottom pan, and attach the nest of sieves to the Yoder-type, wet-sieve shaker. Fill the drum (which holds the set of sieves) with salt-free water at 20–25 °C to a level somewhat below that of the screen in the top sieve of the nest of sieves, when the sieves are in the highest position. Then, lower the nest of sieves to wet the soil for 10 minutes. Bring the nest of sieves to the initial position, and adjust the level of water so that the screen in the top sieve is covered with water in its highest position. Now, switch on the mechanical oscillator to move the nest of sieves up and down with a frequency of 30–35 cycles per minute and a stroke of 3.8 cm. Sieve for 10 minutes. Remove the nest of screens from the water and allow it to drain for some time. Transfer the soil resting on each screen with a stream of distilled water, and brush it into a Büchner funnel having a pre-weighed filter paper and connected to a suction pump. Transfer the soil along with the filter paper into an aluminium can and dry at 105 °C for 24 hours. Weigh the soil to the nearest 0.01 g.

Transfer the oven-dry soil aggregates from all the cans of a set into the dispersion cup. Add dispersing agent (10 ml of 5 percent solution of sodium hexametaphosphate for normal and Ca-saturated soils, or 10 ml of 4 percent solution of sodium hydroxide for acid soil) and enough distilled water to fill the cup to within 4 cm of the rim, and then stir the suspension for 10 minutes. Wash the suspension on an identical set of sieves as used previously by means of a stream of tap-water and a brush, and transfer it to aluminium cans. Weigh and oven-dry the sand in each can in the same manner as above. Calculate the percentage distribution of soil particles (aggregates and the sand) and the sand particles retained on each sieve as follows:

Size distribution of soil particles (aggregate + sand):

➢ Soil particles in each size group (%) =

$$\frac{W_{od}\ (ag + s)\ i \times 100}{W_{od}}$$

➢ Sand particle in each size group (%) =

$$\frac{W_{od}\ (s)\ i \times 100}{W_{od}}$$

where W_{od} is the oven dried weight of the aggregates (*ag*) and sand (*s*), and *i* is the size group.

Cation exchange capacity

The total number of exchangeable cations a soil can hold is called its cation exchange capacity (CEC). The higher the CEC, the more cations it can retain. It can be expressed in terms of milli-equivalents per 100 g of soil (me/100 g) or in centimoles of positive charge per kilogram of soil (cmol/kg), which is numerically equal to me/100 g. The CEC of the soil depends on the kind of clay and OM present.

The apparatus required in order to determine the CEC consists of:

- a centrifuge;
- some 50-ml round-bottom centrifuge tubes;
- a mechanical shaker;
- a flame photometer and accessories that include propane, lithium and sodium standards.

The reagents required are:

- Sodium acetate (NaOAc) 1.0M: Dissolve 136.08 g of sodium acetate trihydrate in distilled water and bring the volume to 1 litre. Adjust the pH to about 8.2.
- Ethanol 95 percent.
- Ammonium acetate (NH_4OAc) 1.0M: Dissolve 77.09 g of ammonium acetate in distilled water and dilute to about 900 ml. Adjust the pH to 7.0 with dilute ammonium hydroxide or acetic acid as required, and make the volume up to 1 litre.
- Standard solution of NaCl: Dissolve 5.845 g of AR-grade NaCl in 1.0M ammonium acetate and make the volume up to 1 litre. It will give 100 me/litre of sodium in stock solution. From this solution take 0, 1, 2, 5, 7.5 and 10 ml and make the volume up to 100 ml each with the ammonium acetate. It will give 0, 1, 2, 5, 7.5 and 10 me/litre of sodium.

The procedure for determining the CEC is:

1. Weigh accurately 5 g of soil, and transfer the sample to a 50-ml centrifuge tube.
2. Add 25 ml of 1.0M sodium acetate solution to the tube, insert the stopper and shake in a mechanical shaker for 5 minutes.
3. Centrifuge at 2 000 rpm for 5 minutes or until the supernatant liquid is clear.
4. Decant the liquid completely and repeat the extraction three more times. Discard the decants.
5. Repeat steps 2–4 with ethanol or isopropyl alcohol until the electrical conductivity (EC) of the decant reads less than 40 mS/cm (it usually takes 4–5 washings).
6. To displace the adsorbed Na, repeat steps 2–4 using the ammonium acetate solution. Collect the decant in a 100-ml volumetric flask fitted with a funnel and filter paper. Make up to volume with ammonium acetate solution.

7. To determine the sodium concentration by flame photometry, prepare a series of Na standard solutions in the range of 0–10 me/litre of Na. Prepare a standard curve by plotting Na concentration on the x-axis and the flamephotometric readings on the y-axis. Feed an unknown sample extract onto the flamephotometer and take the reading, corresponding to which the concentration of Na is read from the standard curve. For better results, add lithium chloride (LiCl) in each standard to yield a final concentration of about 5 me/litre of LiCl.

The ammonium acetate extractable Na that is exchangeable Na in me/100 g soil =

$$\frac{\text{Na conc. of extract in meq/litre (Y)} \times 100}{\text{Wt. of soil in g (5)}} \times \frac{\text{Vol. of extract in ml (100)}}{1\,000} = \frac{Y \times 10}{5} = 2Y$$

This displaced Na is actually a measure of the CEC of the soil. Therefore, the me Na/100 g soil is actually me exchangeable cations (Ca, Mg, Na and K)/100 g soil.

Soil moisture

The gravimetric method of moisture estimation is most widely used where the soil sample is placed in an oven at 105 °C and dried to a constant weight. The difference in weight is considered to be the water present in the soil sample.

The apparatus required in order to determine the soil moisture consists of:

- an aluminium moisture box;
- an oven;
- a desiccator.

The procedure for determining the soil moisture is:

1. Put 100 g of soil sample in the aluminium moisture box and place in the oven after removing the lid of the box.
2. Keep the sample at 105 °C until it attains a constant weight. This may take 24–36 hours.
3. Cool the sample, first in the switched-off oven and then in a desiccator.
4. Weigh the cooled sample. The loss in weight is equal to the moisture contained in 100-g soil sample.

The percentage of moisture is calculated as:

$$\text{Moisture percent} = \frac{\text{Loss in wt.}}{\text{Oven - dry wt. of soil}} \times 100$$

The corresponding moisture correction factor (mcf) for analytical results or the multiplication factor for the amount of sample to be weighed for analysis is:

$$\text{Moisture correction factor} = \frac{100 + \%\ \text{moisture}}{100}$$

Water holding capacity

Veihmeyer and Hendrickson (1931) defined the field capacity or the water holding capacity (WHC) as the amount of water held in the soil after the excess gravitational water has drained away and after the rate of downward movement of water has materially ceased. The stage of field capacity is attained in the field after 48–72 hours of saturation. It is the upper limit of plant-available soil moisture.

The apparatus required in order to determine WHC consists of:

- some polyethylene sheets;
- a spade;
- a soil auger;
- some moisture boxes/cans;
- a balance;
- an oven.

The procedure for determining WHC is:

1. Select a uniform plot measuring 5 m × 5 m.
2. Remove weeds, pebbles, etc., and make bunds around the plot.
3. Fill the plot with sufficient water to saturate the soil completely.
4. Cover the plot area with a polyethylene sheet in order to check evaporation.
5. Take a soil sample from the centre of the plot from the desired layer, starting after 24 hours of saturation, and determine the moisture content daily until the values of successive days are nearly equal.
6. Record the weight as below:
   - weight of empty moisture box = X;
   - weight of moisture box + moist soil = Y;
   - weight of moisture box + oven-dry soil = Z;
   - repeat the above on the next day and so on until a constant Z value is reached.

The calculations are:

- Moisture content in soil = $Y - Z$;
- Weight of oven-dry soil = $Z - X$.

Thus, the percentage of moisture in the soil is given by:

$$\text{Percentage of moisture in soil (1st day)} = \left(\frac{Y - Z}{Z - X}\right) \times 100 = a$$

The percentage of moisture on succeeding days = a_1, a_2, etc.

Plot the daily readings on a graph paper. The lowest reading is taken as a value of field capacity of the soil.

Soil pH

The soil pH is the negative logarithm of the active hydrogen ion (H^+) concentration in the soil solution. It is the measure of soil sodicity, acidity or neutrality. It is a simple but very important estimation for soils as soil pH has a considerable

influence on the availability of nutrients to crops. It also affects microbial population in soils. Most nutrient elements are available in the pH range of 5.5–6.5.

In various chemical estimations, pH regulation is critical. Annex 12 details the specific colours as observed in the presence of various pH indicators and the colour changes due to pH change. The apparatus required in order to measure soil pH consists of:

- a pH meter with a range of 0–14 pH;
- a pipette/dispenser;
- some beakers;
- a glass rod.

The reagents required are:

- Buffer solutions of pH 4, 7 and 9.
- Calcium chloride solution (0.01M): dissolve 14.7 g of $CaCl_2.2H_2O$ in 10 litres of water to obtain 0.01M solution.

The procedure for measuring soil pH is:

1. Calibrate the pH meter, using two buffer solutions, one should be the buffer with neutral pH (7.0) and the other should be chosen based on the range of pH in the soil. Put the buffer solutions in the beakers. Insert the electrode alternately in the beakers containing the two buffer solutions, and adjust the pH. The instrument indicating pH as per the buffers is ready to test the samples.
2. Place 10.0 g of soil sample into a 50-ml or 100-ml beaker, add 20 ml of $CaCl_2$ solution (use water instead of $CaCl_2$ solution throughout the procedure where water is used as a suspension medium).
3. Allow the soil to absorb the $CaCl_2$ solution without stirring, then stir thoroughly for 10 seconds using a glass rod.
4. Stir the suspension for 30 minutes, and record the pH on the calibrated pH meter.

Based on soil pH values, soil reactions are distinguished as per Table 4.

Acid soils need to be limed before they can be put to normal agricultural production. Alkali soils need to be treated with gypsum in order to remove the excessive content of Na.

TABLE 4
Soil reaction ratings

pH range	Soil reaction rating
< 4.6	Extremely acidic
4.6–5.5	Strongly acidic
5.6–6.5	Moderately acidic
6.6–6.9	Slightly acidic
7.0	Neutral
7.1–8.5	Moderately alkaline
> 8.5	Strongly alkaline

Lime requirement

Crop yields are normally high in soils with pH values between 6.0 and 7.5. Lime is added to raise the pH of acid soils, and the amount of lime required to raise the pH to an optimal level is called lime requirement. Various methods are available for determining the lime requirement. The methods discussed here are based on the use of a buffer solution, whose pH undergoes change when treated with acid soils. The pH of the buffer solution decreases gradually as H^+ ion concentration increases. When H^+ increases by 1 me in 100 ml of buffer solution, the pH value decrease by 0.1 unit.

It is necessary to prepare fresh buffer solutions. A 0.05M solution of AR-grade potassium hydrogen phthalate (molecular weight 204.22) gives a pH of 4.0 at 25 °C, and it can be used as a buffer.

Woodruff method

For the method developed by Woodruff (1948), the apparatus required consists of:

- a pH meter;
- some automatic pipettes.

To prepare the reagent (Woodruff's buffer solution), dissolve 10 g of calcium acetate [$Ca(CH_3COOH)_2$], 12 g of paranitrophenol, 10 g of salicylic acid and 1.2 g of sodium hydroxide in distilled water. Adjust the pH to 7.0 with acetic acid or sodium hydroxide, transfer to a 1-litre volumetric flask, and make the volume up to the mark with distilled water.

The procedure for determining lime requirement is:

1. Place 10 g of soil sample in a clean 50-ml beaker.
2. Add 10 ml of distilled water, stir, and wait for 30 minutes.
3. Determine the pH value in soil suspension.
4. If the pH value is less than 5.0 (average of 4.5 and 5.5 to have one value), add 10 ml of Woodruff's buffer solution, stir, and wait for 30 minutes before determining the new pH value.

Table 5 shows the amount of lime required to raise the pH for agricultural purposes.

TABLE 5
Lime required to reduce soil acidity

pH (after buffer)	$CaCO_3$	$Ca(OH)_2$	Marl	Limestone	Dolomite
		(tonnes/ha)			
6.5	6.00	4.68	7.20	9.00	6.54
6.4	7.20	5.62	8.64	10.80	7.85
6.3	8.40	6.55	10.08	12.60	9.16
6.2	9.60	7.49	11.52	14.40	10.46
6.1	10.80	8.42	12.96	16.20	11.77
6.0	12.00	9.36	14.40	18.00	13.08
5.9	13.20	10.30	15.84	19.80	14.39
5.8	14.40	11.23	17.28	21.60	15.70
5.7	15.60	12.17	18.72	23.40	17.00
5.6	16.80	13.10	20.16	25.20	18.31

For practical purposes, the soils with a pH value of 6.6–7.5 are considered nearly neutral. Such soils do not need treating with lime or gypsum. With soils that are acidic and alkaline beyond these limits, the growing of acid-loving and salt-tolerant crops may be considered. As soil amendment is expensive process, only highly acidic soils and the soils with high alkalinity need to be treated with chemical amendments.

Shoemaker method

For the method developed by Shoemaker, McLean and Pratt (1961), the apparatus required consists of:

- a pH meter;
- some automatic pipettes (10 and 20 ml).

To prepare the reagent (extractant buffer), dissolve 1.8 g of nitrophenol, 2.5 ml of triethanolamine, 3.0 g of potassium chromate (K_2CrO_4), 2.0 g of calcium acetate and 53.1 g of calcium chloride in 1 litre of water. Adjust the pH to 7.5 with NaOH.

The procedure for determining lime requirement is:

1. Place 5.0 g of soil sample in a 50-ml beaker.
2. Add 5 ml of distilled water and 10 ml of extractant buffer.
3. Shake continuously for 10 minutes (or intermittently for 20 minutes), and read the pH of the soil buffer suspension with a glass electrode. The pH of the buffer solution is reduced, depending on the extent of soil acidity.

For various levels of measured pH of soil buffer suspension, Table 6 shows the amount of lime required in terms of $CaCO_3$ in order to raise the soil pH to 6.0, 6.4 and 6.8. Lime requirement varies with the type of soils and its CEC.

For practical purposes, the pH of acid soils should not be raised beyond 6.4–6.5.

TABLE 6
Lime requirement for different pH targets

Measured pH of soil buffer suspension	Lime requirement as $CaCO_3$ for bringing soil pH to different levels		
	6.0	6.4	6.8
		(tonnes/ha)	
6.7	2.43	2.92	3.40
6.6	3.40	4.13	4.62
6.5	4.37	5.35	6.07
6.4	5.59	6.56	7.53
6.3	6.65	7.78	8.99
6.2	7.52	8.93	10.21
6.1	8.50	10.21	11.66
6.0	9.48	11.42	13.12

Soil electrical conductivity and gypsum requirement

Soils with a pH value higher than 8.0–8.5 may have the following special features:

- presence of excessive amounts of soluble salts;
- presence of excessive amounts of Na on the exchange complex.

Table 7 summarizes the chemical properties of salt-affected soils.

Such soils are generally not considered suitable for growing most crops unless treated with suitable amendment materials. However, there are salt-tolerant crops that can grow on these soils.

To determine the quality of these soils, the following estimations are required:

- pH (as described above);
- salt content or EC;
- exchangeable Na or gypsum requirement.

Electrical conductivity

Electrical conductivity is a measure of the ionic transport in a solution between the anode and cathode. This means, EC is normally considered to be a measurement of the dissolved salts in a solution. Similar to a metallic conductor, they obey Ohm's law.

As EC depends on the number of ions in the solution, it is important to know the soil/water ratio used. The EC of a soil is conventionally based on the measurement of the EC in the soil solution extract from a saturated soil paste, as it has been found that the ratio of the soil solution in saturated soil paste is about 2–3 times higher than that at field capacity.

As determining the EC of soil solution from a saturated soil paste is cumbersome and requires 400–500 g of soil sample for the determination, a less complex method is normally used. Generally, a 1:2 soil/water suspension is used.

The apparatus required in order to determine EC consists of:

- an EC meter;
- some beakers (25 ml), Erlenmeyer flasks (250 ml) and pipettes;
- filter paper.

To prepare the reagent (0.01M potassium chloride solution), dry a small quantity of AR-grade potassium chloride at 60 °C for 2 hours. Weigh 0.7456 g of it, dissolve it in freshly prepared distilled water, and make the volume up to 1 litre.

TABLE 7
Chemical characteristics of saline, non-saline sodic and saline sodic soils

Soil	EC	Exchangeable sodium percentage	pH
	(dS/m)		
Saline	> 4.0	< 15	< 8.5
Sodic (non-saline)	< 4.0	> 15	> 8.5
Saline sodic	> 4.0	> 15	< 8.5

Source: Richards (1954).

This solution gives an EC of 1 411.8 × 10^{-3}, i.e. 1.412 mS/cm at 25 °C. For best results, select a conductivity standard (KCl solution) close to the sample value.

The procedure for determining EC is:

1. Place 40 g of soil in a 250-ml Erlenmeyer flask, add 80 ml of distilled water, stopper the flask, and shake on a reciprocating shaker for 1 hour. Filter through No. 1 filter paper.
2. Wash the conductivity electrode with distilled water, and rinse with standard KCl solution.
3. Pour some KCl solution into a 25-ml beaker, and dip the electrode in the solution. Adjust the conductivity meter to read 1.412 mS/cm, corrected to 25 °C.
4. Wash the electrode, and dip it into the soil extract.
5. Record the digital display corrected to 25 °C. The EC reading is a measure of the soluble salt content in the extract, and an indication of salinity status of the soil sample (Table 8).

Gypsum requirement

In the estimation of gypsum requirement of saline-sodic/sodic soils (Schoonover, 1952), the attempt is to measure the quantity of gypsum (calcium sulphate) required to replace the Na from the exchange complex. The Na so replaced with the Ca of the gypsum is removed through leaching of the soil. The soils treated with gypsum become dominated with Ca in the exchange complex.

When the Ca of the gypsum is exchanged with Na, there is a reduction in the Ca concentration in the solution. The quantity of Ca reduced is equivalent to the Ca exchanged with Na. It is equivalent to the gypsum requirement of the soil when "Ca" is expressed as $CaSO_4$.

To determine the gypsum requirement, the apparatus required consists of:

- a mechanical shaker;
- a burette (50 ml);
- some pipettes (100 ml and 5 ml).

The reagents required are:

- Saturated gypsum (calcium sulphate) solution: Add 5 g of chemically pure $CaSO_4.2H_2O$ to 1 litre of distilled water. Shake vigorously for 10 minutes using a mechanical shaker and filter through No. 1 filter paper.

TABLE 8
General interpretation of EC values

Soil	EC	Total salt content	Crop reaction
	(mS/cm)	*(%)*	
Salt free	0–2	< 0.15	Salinity effect negligible, except for more sensitive crops
Slightly saline	4–8	0.15–0.35	Yield of many crops restricted
Moderately saline	8–15	0.35–0.65	Only tolerant crops yield satisfactorily
Highly saline	> 15	> 0.65	Only very tolerant crops yield satisfactorily

- 0.01N $CaCl_2$ solution: Dissolve exactly 0.5 g of AR-grade $CaCO_3$ powder in about 10 ml of 1:3 diluted HCl. When completely dissolved, transfer to a 1-litre volumetric flask and dilute to the mark with distilled water. Do not use $CaCl_2$ salt as it is highly hygroscopic.
- 0.01N versenate solution: Dissolve 2.0 g of pure ethylenediamine tetraacetic acid (EDTA) – disodium salt and 0.05 g of magnesium chloride (AR-grade) in about 50 ml of water and dilute to 1 litre. Titrate a portion of this against 0.01N of $CaCl_2$ solution to standardize.
- Eriochrome Black T (EBT) indicator: Dissolve 0.5 g of EBT dye and 4.5 g of hydroxylamine hydrochloride in 100 ml of 95-percent ethanol. Store in a stoppered bottle or flask.
- Ammonium hydroxide-ammonium chloride buffer: Dissolve 67.5 g of pure ammonium chloride in 570 ml of concentrated ammonium hydroxide and dilute to 1 litre. Adjust the pH to 10 using dilute HCl or dilute NH_4OH.

The procedure for determining the gypsum requirement is:

1. Weigh 5 g of air-dry soil in a 250-ml conical flask.
2. Add 100 ml of the saturated gypsum solution. Firmly insert a rubber stopper, and shake for 5 minutes.
3. Filter the contents through No. 1 filter paper.
4. Transfer 5 ml of aliquot of the clear filtrate into a 100 or 150-ml porcelain dish.
5. Add 1 ml of the ammonium hydroxide-ammonium chloride buffer solution and 2–3 drops of EBT indicator.
6. Place 0.01N versenate solution in a 50-ml burette and titrate the contents in the dish until the wine red colour starts to change to sky blue. Volume of versenate used = B.
7. Run a blank using 5 ml of saturated gypsum solution in place of sample aliquot. Volume of versenate solution used = A.

The gypsum requirement (in tonnes per hectare) is given by: $(A - B) \times N \times 382$; where:

- A = millilitres of EDTA (versenate) used for blank titration;
- B = millilitres of ETDA used for soil extract;
- N = normality of EDTA solution.

Organic carbon / organic matter

There are various methods for estimating OM in soil. Loss of weight on ignition can be used as a direct measure of the OM contained in the soil. It can also be expressed as the content of organic C in the soil. It is generally assumed that, on average, OM contains about 58 percent organic C. Organic matter / organic C can also be estimated by volumetric and colorimetric methods. However, the use of potassium dichromate ($K_2Cr_2O_7$) involved in these estimations is considered a limitation because of its hazardous nature. Soil organic matter (SOM) content can be used as an index of N availability (potential of a soil to supply N to plants) because the N content in SOM is relatively constant.

Loss of weight on ignition

The apparatus required using this method consists of:

- a sieve;
- a beaker;
- an oven;
- a muffle furnace.

The procedure is:

1. Weigh 5.0–10.0 g (to the nearest 0.01 g) of sieved (2 mm) soil into an ashing vessel (50-ml beaker or other suitable vessel).
2. Place the ashing vessel with soil in a drying oven set at 105 °C and dry for 4 hours. Remove the ashing vessel from the drying oven and place in a dry atmosphere. When cooled, weigh to the nearest 0.01 g. Place the ashing vessel with soil into a muffle furnace, and bring the temperature to 400 °C. Ash in the furnace for 4 hours. Remove the ashing vessel from the muffle furnace, cool in a dry atmosphere, and weigh to the nearest 0.01 g.

The percentage of OM is given by:

$$\text{Percent organic matter (OM)} = \frac{(W_1 - W_2)}{W_1} \times 100$$

where:

- W_1 is the weight of soil at 105 °C;
- W_2 is the weight of soil at 400 °C.

The percent of organic C is given by: % OM × 0.58.

Volumetric method

The apparatus required for the volumetric method (Walkley and Black, 1934) consists of:

- a conical flask (500 ml);
- some pipettes (2, 10 and 20 ml);
- a burette (50 ml).

The reagents required are:

- Phosphoric acid – 85 percent.
- Sodium fluoride solution – 2 percent.
- Sulphuric acid – 96 percent containing 1.25 percent of Ag_2SO_4.
- Standard 0.1667M $K_2Cr_2O_7$: Dissolve 49.04 g of $K_2Cr_2O_7$ in water and dilute to 1 litre.
- Standard 0.5M $FeSO_4$ solution: Dissolve 140 g of ferrous sulphate or 196.1 g of $FeSO_4.(NH_4)_2.6H_2O$ in 800 ml of water, add 20 ml of concentrated H_2SO_4 and make the volume up to 1 litre.
- Diphenylamine indicator: Dissolve 0.5 g of reagent-grade diphenylamine in 20 ml of water and 100 ml of concentrated H_2SO_4.

The procedure is:

1. Weigh 1.0 g of the prepared soil sample in a 500-ml conical flask.
2. Add 10 ml of 0.1667M $K_2Cr_2O_7$ solution and 20 ml of concentrated H_2SO_4 containing Ag_2SO_4.

3. Mix thoroughly and allow the reaction to complete for 30 minutes.
4. Dilute the reaction mixture with 200 ml of water and 10 ml of H_3PO_4.
5. Add 10 ml of NaF solution and 2 ml of diphenylamine indicator.
6. Titrate the solution with standard 0.5M $FeSO_4$ solution to a brilliant green colour.
7. Run a blank without sample simultaneously.

The percentage of organic C is given by:

$$\frac{10\,(S - T) \times 0.003}{S} \times \frac{100}{\text{Wt. of soil}}$$

As 1 g of soil is used, this equation simplifies to:

$$\frac{3\,(S - T)}{S}$$

where:

- S = millilitres of $FeSO_4$ solution required for blank;
- T = millilitres of $FeSO_4$ solution required for soil sample;
- 0.003 = weight of C (1 000 ml 0.1667M $K_2Cr_2O_7$ = 3 g C. Thus, 1 ml 0.1667M $K_2Cr_2O_7$ = 0.003 g C).

Organic C recovery is estimated to be about 77 percent. Therefore, the actual amount of organic C (Y) will be:

$$\text{Percent value of organic carbon obtained} \times \frac{100}{77}$$

Or: percentage value of organic C × 1.3.

Percent OM = Y × 1.724 (organic matter contains 58 percent organic C, hence 100/58 = 1.724).

Published organic C to total OM conversion factors for surface soils vary from 1.724 to 2.0. A value of 1.724 is commonly used. However, where possible, the appropriate factor should be determined experimentally for each type of soil.

Colorimetric method

The apparatus required for the colorimetric method (Datta, Khera and Saini, 1962) consists of:

- a spectrophotometer;
- some conical flasks (100 ml);
- some pipettes (2, 5 and 10 ml).

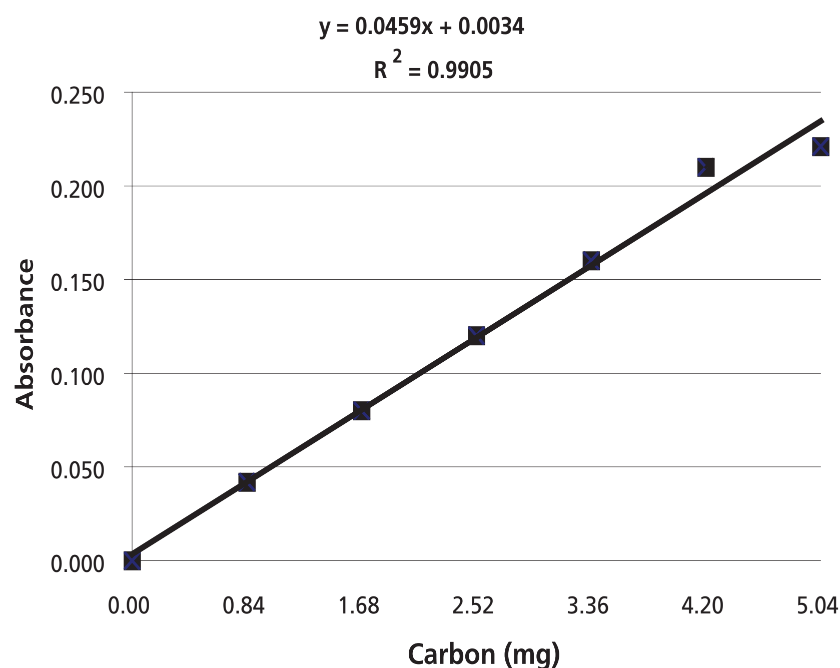
The reagents required are:

- Standard potassium dichromate 0.1667M.
- Concentrated sulphuric acid containing 1.25 percent of Ag_2SO_4.
- Sucrose (AR-grade).

The procedure is:

1. Preparation of standard curve: Sucrose is used as a primary standard C source. Place different quantities of sucrose (1–20 mg) in 100-ml flasks. Add 10 ml of standard $K_2Cr_2O_7$ and 20 ml of concentrated H_2SO_4 in each flask. Swirl the flasks, and leave for 30 minutes. Prepare a blank in the same way without adding sucrose. A green colour develops, which is read on spectrophotometer at 660 nm, after adjusting the blank to zero. Plot the reading so obtained against milligrams of sucrose as C source (C = weight of sucrose × 0.42 – because the C content of sucrose is 42 percent) or against milligrams of C directly. As an example, Figure 2 shows a standard curve as prepared for estimating organic C by the authors of this publication while setting up a soil testing laboratory. It shows the accuracy of the method (r^2 is as high as 0.991). For convenience, the curve is shown directly against C content, which has been derived from milligrams of sucrose used in preparing the standard curve.
2. Place 1 g of soil in a 100-ml conical flask.
3. Add 10 ml of 0.1667M $K_2Cr_2O_7$ and 20 ml of concentrated H_2SO_4 containing 1.25 percent of Ag_2SO_4.

FIGURE 2
Standard curve for organic carbon on spectrophotometer



4. Stir the reaction mixture and allow it to stand for 30 minutes.
5. The green colour of chromium sulphate so developed is read on a spectrophotometer at 660 nm after setting the blank, prepared in the similar manner, at zero.

The C content of the sample is found from the standard curve, which shows the C content (milligrams of C vs spectrophotometer readings as absorbance):

- Percent C = milligrams of C observed × 100 / 1 000 (observed reading is for 1 g soil, expressed as milligrams).
- Percent OM = %C × 1.724.

Total nitrogen

Total N includes all forms of inorganic N, such as NH_4, NO_3 and NH_2 (urea), and the organic N compounds such as proteins, amino acids and other derivatives. Depending on the form of N present in a particular sample, a specific method is to be adopted for determining the total N value. While organic N materials can be converted into simple inorganic ammoniacal salt by digestion with sulphuric acid, for reducing nitrates into ammoniacal form, the modified Kjeldahl method is adopted with the use of salicylic acid or Devarda's alloy. At the end of digestion, all organic and inorganic salts are converted into ammonium form, which is distilled and estimated by using standard acid.

As the precision of the method depends on complete conversion of organic N into NH_4-N, the digestion temperature and time, the solid–acid ratio and the type of catalyst used have an important bearing on the method. The ideal temperature for digestion is 320–370 °C. At a lower temperature, the digestion may not be complete, while above 410 °C, loss of NH_3 may occur. The salt–acid (weight–volume) ratio should not be less than 1:1 at the end of digestion. Commonly used catalysts to accelerate the digestion process are $CuSO_4$ and mercury (Hg). Potassium sulphate is added to raise the boiling point of the acid so that loss of acid by volatilization is prevented.

The apparatus required for this method consists of:

- a Kjeldahl digestion and distillation unit;
- some conical flasks;
- some burettes;
- some pipettes.

The reagents required are:

- Sulphuric acid (93–98 percent).
- Copper sulphate ($CuSO_4.H_2O$) (AR-grade).
- Potassium sulphate or anhydrous sodium sulphate (AR-grade).
- 35-percent sodium hydroxide solution: Dissolve 350 g of solid NaOH in water and dilute to 1 litre.
- 0.1M NaOH: Prepare 0.1M NaOH by dissolving 4.0 g of NaOH in water and make the volume up to 1 litre. Standardize against 0.1N potassium hydrogen phthalate or standard H_2SO_4.

- 0.1M HCl or 0.05M H_2SO_4: Prepare approximately the standard acid solution and standardize against 0.1M sodium carbonate.
- Methyl red indicator.
- Salicylic acid for reducing NO_3 to NH_4, if present in the sample.
- Devarda's alloy for reducing NO_3 to NH_4, if present in the sample.

The procedure is

1. Weigh 1 g of soil sample. Place in a Kjeldahl flask.
2. Add 0.7 g of copper sulphate, 1.5 g of K_2SO_4 and 30 ml of H_2SO_4.
3. Heat gently until frothing ceases. If necessary, add a small amount of paraffin or glass beads to reduce frothing.
4. Boil briskly until the solution is clear and then continue digestion for at least 30 minutes.
5. Remove the flask from the heater and cool, add 50 ml of water, and transfer to a distilling flask.
6. Place accurately 20–25 ml of standard acid (0.1M HCl or 0.05M H_2SO_4) in the receiving conical flask so that there will be an excess of at least 5 ml of the acid. Add 2–3 drops of methyl red indicator. Add enough water to cover the end of the condenser outlet tubes.
7. Run tap-water through the condenser.
8. Add 30 ml of 35-percent NaOH in the distilling flask in such a way that the contents do not mix.
9. Heat the contents to distil the ammonia for about 30–40 minutes.
10. Remove the receiving flask and rinse the outlet tube into the receiving flask with a small amount of distilled water.
11. Titrate excess acid in the distillate with 0.1M NaOH.
12. Determine blank on reagents using the same quantity of standard acid in a receiving conical flask.

The calculation is:

$$\text{Percent N} = \frac{1.401\left[(V_1 M_1 - V_2 M_2) - (V_3 M_1 - V_4 M_2)\right]}{W} \times df$$

where:

- V_1 – millilitres of standard acid put in receiving flask for samples;
- V_2 – millilitres of standard NaOH used in titration;
- V_3 – millilitres of standard acid put in receiving flask for blank;
- V_4 – millilitres of standard NaOH used in titrating blank;
- M_1 – molarity of standard acid;
- M_2 – molarity of standard NaOH;
- W – weight of sample taken (1 g);
- df – dilution factor of sample (if 1 g was taken for estimation, the dilution factor will be 100).

Note: 1 000 ml of 0.1M HCl or 0.05M H_2SO_4 corresponds to 1.401 g of N.

The following precautions should be observed:

- The material should not solidify after digestion.

- No NH_4 should be lost during distillation.
- If the indicator changes colour during distillation, determination must be repeated using either a smaller sample weight or a larger volume of standard acid.

Mineralizable nitrogen

In the case of soils, mineralizable N (also organic C) is estimated as an index of available N content and not the total N content (Subbiah and Asija, 1956). The easily mineralizable N is estimated using alkaline $KMnO_4$, which oxidizes and hydrolyses the OM present in the soil. The liberated ammonia is condensed and absorbed in boric acid, which is titrated against standard acid. Because of its rapidity and reproducibility, the method has been widely adopted. However, as the process of oxidative hydrolysis is a progressive one, a uniform time and heating temperature should be respected for best results. The use of glass beads checks bumping, while liquid paraffin checks frothing during heating (as recommended in total N estimation by the Kjeldahl method).

The apparatus required in order to estimate mineralizable N consists of:

- a nitrogen distillation unit, preferably with six regulating heating elements;
- some conical flasks, pipettes, burettes, etc.

The reagents required are:

- 0.32 percent $KMnO_4$: Dissolve 3.2 g of $KMnO_4$ in water and make the volume up to 1 litre.
- 2.5 percent NaOH: Dissolve 25 g of sodium hydroxide pellets in water and make the volume up to 1 litre.
- 2 percent boric acid: Dissolve 20 g of boric acid powder in warm water by stirring and dilute to 1 litre.
- Mixed indicator: Dissolve 0.066 g of methyl red and 0.099 g of bromocresol green in 100 ml of ethyl alcohol. Add 20 ml of this mixed indicator to each litre of 2 percent boric acid solution.
- 0.1M potassium hydrogen phthalate: Dissolve 20.422 g of the salt in water and dilute to 1 litre. This is a primary standard and does not require standardization.
- 0.02M H_2SO_4: Prepare approximately 0.1M H_2SO_4 by adding 5.6 ml of concentrated H_2SO_4 to about 1 litre of distilled water. From this, prepare 0.02M H_2SO_4 by diluting a suitable volume (20 ml made to 100 ml) with distilled water. Standardize it against 0.1M NaOH solution.
- 0.1M NaOH. Dissolve 4 g of NaOH in 100 ml of distilled water. Standardize against potassium hydrogen phthalate.

The procedure is:

1. Weigh 20 g of soil sample in an 800-ml Kjeldahl flask.
2. Moisten the soil with about 10 ml of distilled water, wash down the soil, if any, adhering to the neck of the flask.
3. Add 100 ml of 0.32 percent $KMnO_4$ solution.
4. Add a few glass beads or broken pieces of glass rod.

5. Add 2–3 ml of paraffin liquid, avoiding contact with the upper part of the neck of the flask.
6. Measure 20 ml of 2 percent boric acid containing mixed indicator in a 250-ml conical flask and place it under the receiver tube. Dip the receiver tube in the boric acid.
7. Run tap-water through the condenser.
8. Add 100 ml of 2.5 percent NaOH solution, and immediately attach to the rubber stopper fitted in the alkali trap.
9. Switch the heaters on and continue distillation until about 100 ml of distillate is collected.
10. First, remove the conical flask containing distillate and then switch off the heaters to avoid back suction.
11. Titrate the distillate against 0.02M H_2SO_4 in a burette until a pink colour starts to appear.
12. Run a blank without soil.
13. Carefully remove the Kjeldahl flask after cooling and drain the contents in the sink.

The calculation for estimating mineralizable N is:

$$\text{Volume of acid used to neutralize ammonia in the sample} = A - B \text{ ml}$$

$$\text{N content in the test sample} = (A - B) \times 0.56 \text{ mg}$$

$$\text{Percent N} = (A - B) \times 0.56 \times 5$$

where:

- A = volume of 0.02M H_2SO_4 used in titration against ammonia absorbed in boric acid;
- B = volume of 0.02M sulphuric acid used in blank titration.

1 000 ml of 1M H_2SO_4 = 28 g N; thus, 1 ml of 0.02M sulphuric acid = 0.56 mg N. The weight of the soil sample is 20 g, thus, the factor for converting into percent N = 100/20 = 5.

It is important to remember that:

- All the joints of the Kjeldahl apparatus should be checked in order to prevent any leakage and loss of ammonia.
- Hot Kjeldahl flasks should neither be washed immediately with cold water nor allowed to cool for long (to avoid deposits from settling at the bottom, which are difficult to remove).
- If frothing occurs and passes through to the boric acid, such samples should be discarded and fresh distillation done.
- The opening of ammonia bottles in the laboratory should be strictly prohibited while distillation is on. The titration should be carried out in an ammonia-free atmosphere.
- If the titration is not to be carried out immediately, the distillate should be stored in ammonia-free cupboards after tightly stoppering the flasks.

Inorganic N – NO_3^- and NH_4^+

Inorganic N in soil is present predominantly as NO_3^--N and NH_4^+-N. Nitrite is seldom present in detectable amounts, and its determination is normally unwarranted except in neutral to alkali soils following the application of NH_4 or NH_4-forming fertilizers.

Nitrate is highly soluble in water, and a number of extractants including water have been used. These include saturated 0.35 percent $CaSO_4$ $2H_2O$ solution, 0.03M NH_4F, 0.015M H_2SO_4, 0.01M $CaCl_2$, 0.5M $NaHCO_3$ (pH 8.5), 0.01M $CuSO_4$, 0.01M $CuSO_4$ containing Ag_2SO_4, and 2.0M KCl.

Exchangeable NH_4 is defined as NH_4 that can be extracted at room temperature with a neutral K-salt solution. Various molarities have been used, such as 0.05M K_2SO_4, 0.1M KCl, 1.0M KCl, and 2.0M KCl.

The potential of a soil to mineralize N as measured by N-availability indices (OM, organic C and even total N) is fairly constant from year to year, making it unnecessary to conduct this type of determination each year. However, it is still necessary to take into consideration the initial amount of available N (inorganic N: NO_3-N and/or NH_4-N) in the rootzone at or near planting time for better prediction of N fertilizer needs. This type of test must be conducted each year, especially where there is the possibility of residual inorganic N remaining from a previous application or fallow period.

The methods for the determination of NO_3-N and NH_4-N are more diverse than the methods of extraction (Keeney and Nelson, 1982). They range from specific ion electrode to colorimetric techniques, microdiffusion, steam distillation, and flow injection analysis. Steam distillation is still a preferred method when using ^{15}N. However, for routine analysis, this guide details the phenoldisulphonic acid method for NO_3 and the indophenol blue method for NH_4 estimation.

Nitrate by phenoldisulphonic acid method

A major difficulty in estimating NO_3 in soils by colorimetric methods is obtaining a clear colourless extract with low contents of organic and inorganic substances, which interfere with the colorimetric method. In arid and salt-affected soils, chloride is the major anion that interferes with colour development of the phenoldisulphonic acid method. Therefore, if the chloride concentration is more than 15 µg/g soil, it should be removed before analysis by the use of Ag_2SO_4 to precipitate chloride as AgCl. The Ag_2SO_4 is added to the extract or to the reagent used for extraction, and the AgCl is removed by filtration or centrifugation after precipitation of the excess Ag_2SO_4 by a basic reagent such as $Ca(OH)_2$ or $MgCO_3$. It is necessary to remove the excess Ag^+ before analysis of the extract because it also interferes with the phenoldisulphonic acid method of determining NO_3.

The apparatus required using the method consists of:

- a reciprocating shaker;
- a heavy-duty hotplate;
- a spectrophotometer;

- a dispenser;
- an Erlenmeyer flask;
- some beakers;
- a glass rod.

The reagents required are:

- Phenoldisulphonic acid (phenol 2,4-disulphonic acid): Transfer 70 ml of pure liquid phenol (carbolic acid) to an 800-ml Kjeldahl flask. Add 450 ml of concentrated H_2SO_4 while shaking. Add 225 ml of fuming H_2SO_4 (13–15 percent SO_3). Mix well. Place the Kjeldahl flask (loosely stoppered) in a beaker containing boiling water and heat for 2 hours. Store the resulting phenoldisulphonic acid [$C_6H_3OH(HSO_3)_2$] solution in a glass-stoppered bottle.
- Dilute ammonium hydroxide solution (about 7.5M NH_4OH): Mix one part NH_4OH (specific gravity 0.90) with one part H_2O.
- Copper sulphate solution (0.5M): Dissolve 125 g of $CuSO_4.5H_2O$ in 1 litre of distilled water.
- Silver sulphate solution (0.6 percent): Dissolve 6.0 g of Ag_2SO_4 in 1 litre of distilled water. Heat or shake well until all salt is dissolved.
- Nitrate-extracting solution: Mix 200 ml of 0.5M copper sulphate solution and 1 litre of 0.6 percent silver sulphate solution and dilute to 10 litres with water. Mix well.
- Standard nitrate solution (100 µg NO_3-N/ml, stock solution): Dissolve 0.7221 g of KNO_3 (oven dried at 105 °C) in water and dilute to 1 litre. Mix thoroughly.
- Standard nitrate solution (10 µg NO_3-N/ml, working solution): Dilute 100 ml of 100 µg NO_3-N/ml stock solution to 1 litre with water. Mix well.
- Calcium hydroxide: AR-grade powder (free of NO_3).
- Magnesium carbonate: AR-grade powder (free of NO_3).

The procedure is:

1. Place about 5 g of soil in an Erlenmeyer flask.
2. Add 25 ml of nitrate-extracting solution.
3. Shake contents for 10 minutes.
4. Add about 0.2 g of $Ca(OH)_2$ and shake for 5 minutes.
5. Add about 0.5 g of $MgCO_3$ and shake for 10–15 minutes.
6. Allow to settle for a few minutes.
7. Filter through No. 42 filter paper.
8. Pipette 10 ml of clear filtrate into a 100-ml beaker. Evaporate to dryness on a hotplate at low heat in a fumehood (free of HNO_3 fumes). Do not continue heating beyond dryness.
9. When completely dry, cool residue, add 2 ml of phenoldisulphonic acid rapidly (from a burette having the tip cut off), covering the residue quickly. Rotate the beaker so that the reagent comes into contact with all residual salt. Allow to stand for 10–15 minutes.

10. Add 16.5 ml of cold water. Rotate the beaker to dissolve residue (or stir with a glass rod until all residue is in solution).
11. Once the beaker is cool, add 15 ml of dilute NH_4OH slowly until the solution is distinctly alkaline as indicated by the development of a stable yellow colour.
12. Add 16.5 ml of water (volume becomes 50 ml). Mix thoroughly.
13. Read the concentration of NO_3-N at 415 nm, using the standard curve.
14. Preparation of standard curve: Place 0, 2, 5, 8, and 10 ml of the 10 µg NO_3/ml working solution in separate 100-ml beakers, add 10 ml NO_3-extracting solution and evaporate to dryness. Follow steps 9–13, using these standard solutions with 0, 0.4, 1.0, 1.6 and 2.0 µg NO_3-N/ml. Prepare a standard curve to be used for estimation of NO_3 in the sample.

The calculation is:

$$NO_3^- - N \text{ in test soln.} (\mu g/m) = \frac{\text{Vol. after colour develop. (ml)}}{\text{Vol. evaporated (ml)}} \times \frac{\text{Vol. of extracting soln. (ml)}}{\text{Wt. of oven} - \text{dried soil (g)}}$$

Ammonium by indophenol blue method

Phenol reacts with NH_4 in the presence of an oxidizing agent such as hypochlorite to form a coloured complex in alkaline condition. The addition of sodium nitroprusside as a catalyst in the reaction between phenol and NH_4 increases the sensitivity of the method considerably. The addition of EDTA is necessary in order to complex divalent and trivalent cations present in the extract. Otherwise, it forms precipitate at a pH of 11.4–12 used for colour development, and this turbidity would interfere with formation of the phenol–NH_4 complex.

The first step is the extraction of exchangeable ammonium.

The apparatus required consists of:

- an Erlenmeyer flask;
- a volumetric flask;
- a shaker;
- a spectrophotometer.

The reagents required are:

- Potassium chloride (KCl) solution, 2M: Dissolve 150 g of AR-grade KCl in 1 litre of distilled water.
- Standard ammonium (NH_4^+) solution: Dissolve 0.4717 g of ammonium sulphate $(NH_4)_2SO_4$ in water, and dilute to a volume of 1 litre. If pure dry $(NH_4)_2SO_4$ is used, the solution contains 100 µg of NH_4-N/ml. Store the solution in a refrigerator. Immediately before use, dilute 4 ml of this stock NH_4^+ solution to 200 ml. The resulting working solution contains 2 µg of NH_4-N/ml. Accordingly, for the standard curve, prepare various concentrations of standard solution.
- Phenol-nitroprusside reagent: Dissolve 7 g of phenol and 34 mg of sodium nitroprusside [disodium pentacyanonitrosylferrate, $Na_2Fe(CN)_5NO.2H_2O$]

in 80 ml of NH_4^+-free water and dilute to 100 ml. Mix well, and store in a dark-coloured bottle in the refrigerator.

- Buffered hypochlorite reagent: Dissolve 1.480 g of sodium hydroxide (NaOH) in 70 ml of NH_4^+-free water, add 4.98 g of sodium monohydrogen phosphate (Na_2HPO_4) and 20 ml of sodium hypochlorite (NaOCl) solution (5–5.25 percent NaOCl). Use less or more hypochlorite solution if the concentration is higher or lower, respectively, than that indicated above. Check the pH to ensure a value between 11.4 and 12.2. Add a small amount of additional NaOH if required to raise the pH. Dilute to a final volume of 100 ml.
- EDTA reagent: Dissolve 6 g of EDTA disodium salt in 80 ml of deionized water, adjust to pH 7, mix well, and dilute to a final volume of 100 ml.

The procedure for the extraction is:

1. Place 10 g of soil in a 250-ml wide-mouth Erlenmeyer flask and add 100 ml of 2M KCl.
2. Insert stopper, and shake the flask on a mechanical shaker for 1 hour.
3. Allow the soil–KCl suspension to settle (about 30 minutes) until the supernatant is clear.
4. If the KCl extract cannot be analysed within 24 hours, then filter the soil–KCl suspension (No. 42 filter paper) and store in the refrigerator.

Aliquots from this extract are used for the NH_4 estimation.

The procedure for the estimation is:

1. Pipette an aliquot (not more than 5 ml) of the filtered 2M KCl extract containing between 0.5 and 12 µg of NH_4-N into a 25-ml volumetric flask. Aliquots of ≤ 3 ml normally contain sufficient NH_4-N for quantification.
2. Add 1 ml of the EDTA reagent, and mix the contents of the flask.
3. Allow the contents to stand for 1 minute, then add 2 ml of the phenol-nitroprusside reagent, followed by 4 ml of the buffered hypochlorite reagent, and immediately dilute the flask to volume (25 ml) with NH_4^+-free water, and mix well.
4. Place the flask in a water-bath maintained at 40 °C for 30 minutes.
5. Remove the flask from the bath, cool to room temperature, and determine the absorbance of the coloured complex at a wavelength of 636 nm against a reagent blank solution.
6. Determine the NH_4-N concentration of the sample by reference to a calibration curve plotted from the results obtained with 25-ml standard samples containing 0, 2, 4, 6, 8, 10, and 12 µg of NH_4-N/ml.
7. To prepare this curve, add an appropriate amount of 2M KCl solution (same volume as that used for aliquots of soil extract, i.e. about 5 ml) to a series of 25-ml volumetric flasks. Add 0, 1, 2, 3, 4, 5 and 6 ml of the 2 µg NH_4-N/ml solution to the flasks, and measure the intensity of blue colour developed with these standards by the procedure described above for the analysis of unknown extracts.

The calculation is (NH_4-N in the sample as noted from the standard curve = *A* [µg/ml]):

$$\mu g \text{ of } NH_4\text{- N in 1 g soil} = \frac{A \times 100 \text{(total vol. of extract)}}{5 \text{(vol. of extract estimated)}} \times \frac{1}{10 \text{(wt. of soil)}} = 2A$$

where:

- weight of the soil taken for estimation = 10 g;
- total volume of extract = 100 ml;
- volume of extract taken for estimation = 5 ml.

Available phosphorus

The two methods most commonly used for determining the available P in soils are: Bray's Method No. 1 for acid soils; and Olsen's method for neutral and alkali soils.

In these methods, specific coloured compounds are formed with the addition of appropriate reagents in the solution, the intensity of which is proportionate to the concentration of the element being estimated. The colour intensity is measured spectrophotometrically. In spectrophotometric analysis, light of definite wavelength (not exceeding, say, 0.1–1.0 nm in bandwidth) extending to the ultraviolet region of the spectrum constitutes the light source. The photoelectric cells in the spectrophotometer measure the light transmitted by the solution.

A spectrophotometer, as its name implies, is really two instruments in one cabinet – a spectrometer and a photometer. A spectrometer is a device for producing coloured light of any selected colour (or wavelength) and, when employed as part of a spectrophotometer, it is usually termed a monochromator and is generally calibrated in wavelengths. A photometer is a device for measuring the intensity of the light. When incorporated in a spectrophotometer, it is used to measure the intensity of the monochromatic beam produced by the associated monochromator. Generally, the photometric measurement is made first with a reference liquid and then with a coloured sample contained in similar cells interposed in the light beam. The ratio of the two intensity measurements is a measure of the opacity of the sample at the wavelength of the test.

Table 9 lists the approximate wavelength ranges of complementary colours. White light covers the entire visible spectrum (400–760 nm).

Bray's Method No. 1

The apparatus required for Bray's Method No. 1 (Bray and Kurtz, 1945) for acid soils consists of:

- a spectrophotometer;
- some pipettes (2, 5, 10 and 20 ml);
- some beakers/flasks (25, 50, 100 and 500 ml).

The reagents required are:

- Bray's Extractant No. 1 (0.03M NH_4F in 0.025M HCL): Dissolve 2.22 g of NH_4F in 200 ml of distilled water, filter, and add to the filtrate 1.8 litres of

TABLE 9
Wavelengths and corresponding colour ranges

Wavelength	Hue (transmitted)*	Complementary hue of the solution
(nm)		
< 400	Ultraviolet	
400–435	Violet	Yellow green
435–480	Blue	Yellow
480–490	Greenish blue	Orange
490–500	Bluish green	Red
500–560	Green	Purple
560–580	Yellowish green	Violet
580–595	Yellow	Blue
595–610	Orange	Greenish blue
610–750	Red	Bluish green
> 760	Infrared	

* Hue is one of the three main attributes of perceived colour.
Source: Vogel, 1961.

water containing 4 ml of concentrated HCl, make the volume up to 2 litres with distilled water.

- Molybdate reagent: Dissolve 1.50 g of $(NH_4)_2MoO_4$ in 300 ml of distilled water. Add the solution to 350 ml of 10M HCl solution gradually with stirring. Dilute to 1 litre with distilled water.
- Stannous chloride solution (stock solution): Dissolve 10 g of $SnCl_2\,2H_2O$ in 25 ml of concentrated HCl. Add a piece of pure metallic tin, and store the solution in a glass stoppered bottle.
- Stannous chloride solution (working solution): Dilute 1 ml of the stock solution of stannous chloride to 66.0 ml with distilled water just before use. Prepare fresh dilute solution every working day.

The procedure is:

1. Preparation of the standard curve: Dissolve 0.2195 g of pure dry KH_2PO_4 in 1 litre of distilled water. This solution contains 50 µg P/ml. Preserve this as a stock standard solution of phosphate. Take 10 ml of this solution and dilute it to 0.5 litres with distilled water. This solution contains 1 µg P/ml (0.001 mg P/ml). Put 0, 1, 2, 4, 6 and 10 ml of this solution in separate 25-ml flasks. Add to each flask, 5 ml of the extractant solution, 5 ml of the molybdate reagent; and dilute with distilled water to about 20 ml. Add 1 ml of dilute $SnCl_2$ solution, shake again and dilute to the 25-ml mark. After 10 minutes, read the blue colour of the solution on the spectrophotometer at a wavelength of 660 nm. Plot the absorbance reading against "µg P" and connect the points.
2. Extraction: Add 50 ml of the Bray's Extractant No. 1 to a 100-ml conical flask containing 5 g of soil sample. Shake for 5 minutes and filter.
3. Development of colour: Take 5 ml of the filtered soil extract with a bulb pipette in a 25-ml measuring flask; deliver 5 ml of the molybdate reagent with an automatic pipette, dilute to about 20 ml with distilled water, shake

and add 1 ml of the dilute $SnCl_2$ solution with a bulb pipette. Fill to the 25-ml mark and shake thoroughly. Read the blue colour after 10 minutes on the spectrophotometer at 660 nm after setting the instrument to zero with the blank prepared similarly but without the soil.

The calculation is:

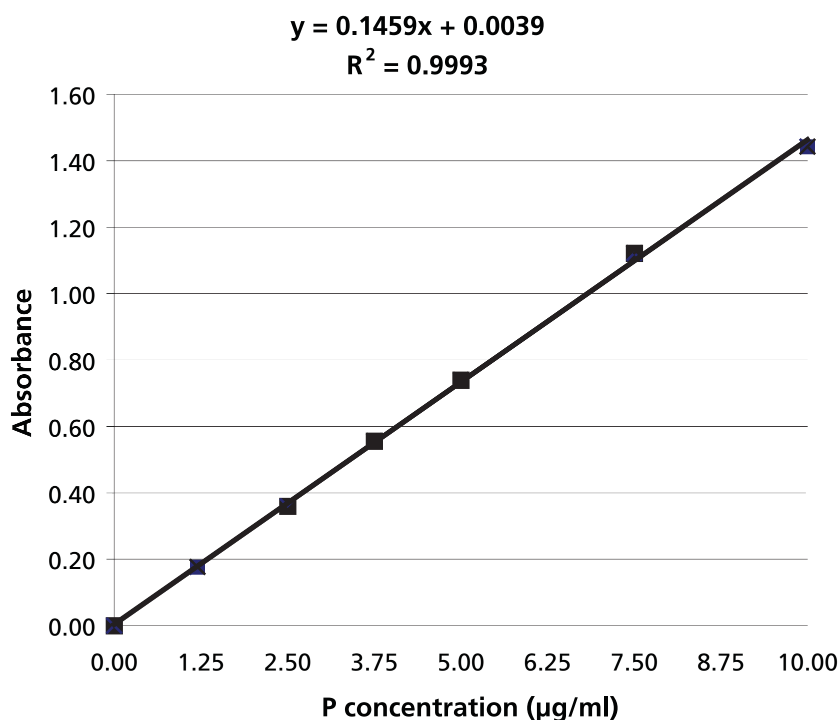
$$P\,(kg/ha) = \frac{A}{1\,000\,000} \times \frac{50}{5} \times \frac{2\,000\,000}{5} = 4A$$

where:

- weight of the soil taken = 5 g;
- volume of the extract = 50 ml;
- volume of the extract taken for estimation = 5 ml;
- amount of P observed in the sample on the standard curve = *A* (µg);
- weight of 1 ha of soil down to a depth of 22 cm is taken as 2 million kg.

As an example, Figure 3 shows a standard curve prepared by the authors of this publication for estimation of available P by Bray's Method No. 1 while establishing a soil testing laboratory.

FIGURE 3
Standard curve for P on spectrophotometer



Olsen's method

The apparatus required for Olsen's method (Olsen *et al.*, 1954) for alkali soils is the same as that for Bray's Method No. 1 (above).

The reagents required are:

- Bicarbonate extractant: Dissolve 42 g of sodium bicarbonate in 1 litre of distilled water and adjust the pH to 8.5 by addition of dilute NaOH or HCl. Filter as necessary.
- Activated P-free carbon.
- Molybdate reagent: Same as for the Bray's Method No. 1 (above).
- Stannous chloride solution: Same as for Bray's Method No. 1 (above).

The procedure is:

1. Preparation of the standard curve: proceed as for Bray's Method No. 1 (above).
2. Extraction: Add 50 ml of the bicarbonate extractant to a 100-ml conical flask containing 2.5 g of soil sample. Add 1 g of activated carbon. Shake for 30 minutes on the mechanical shaker, and filter.
3. Development of colour: proceed as for Bray's Method No. 1 (above).

The calculation is the same as described for Bray's Method No. 1 (above).

In spite of all precautions, the intensity of blue colour changes slightly with every batch of molybdate reagent. It is imperative to check the standard curve every day by using 2–3 dilutions of the standard phosphate solution. If the standard curve does not tally, draw a new standard curve with fresh molybdate reagent.

Available potassium

Potassium present in the soil is extracted with neutral ammonium acetate of 1 molarity. This is considered as plant-available K in the soils. It is estimated with the help of a flame photometer (Toth and Prince, 1949).

The apparatus required consists of:

- a multiple dispenser or automatic pipette (25 ml);
- some flasks and beakers (100 ml);
- a flame photometer.

The reagents required are:

- Molar neutral ammonium acetate solution: Dissolve 77 g of ammonium acetate ($NH_4C_2H_3O_2$) in 1 litre of water. Check the pH with bromothymol blue or with a pH meter. If not neutral, add either ammonium hydroxide or acetic acid as per the need in order to neutralize it to pH 7.0.
- Standard potassium solution: Dissolve 1.908 g of pure KCl in 1 litre of distilled water. This solution contains 1 mg K/ml. Take 100 ml of this solution and dilute to 1 litre with ammonium acetate solution. This gives 0.1 mg K/ml as a stock solution.
- Working potassium standard solutions: Take 0, 5, 10, 15 and 20 ml of the stock solution and dilute each volume separately to 100 ml with the molar ammonium acetate solution. These solutions contain 0, 5, 10, 15 and 20 µg K/ml, respectively.

The procedure is:

1. Preparation of the standard curve: Set up the flame photometer by atomizing 0 and 20 µg K/ml solutions alternatively to readings of 0 and 100. Atomize intermediate working standard solutions and record the readings. Plot these readings against the respective K contents and connect the points with a straight line to obtain a standard curve.
2. Extraction: Add 25 ml of the ammonium acetate extractant to a conical flask fixed in a wooden rack containing 5 g of soil sample. Shake for 5 minutes and filter.
3. Determine the potash in the filtrate with the flame photometer.

The calculation is:

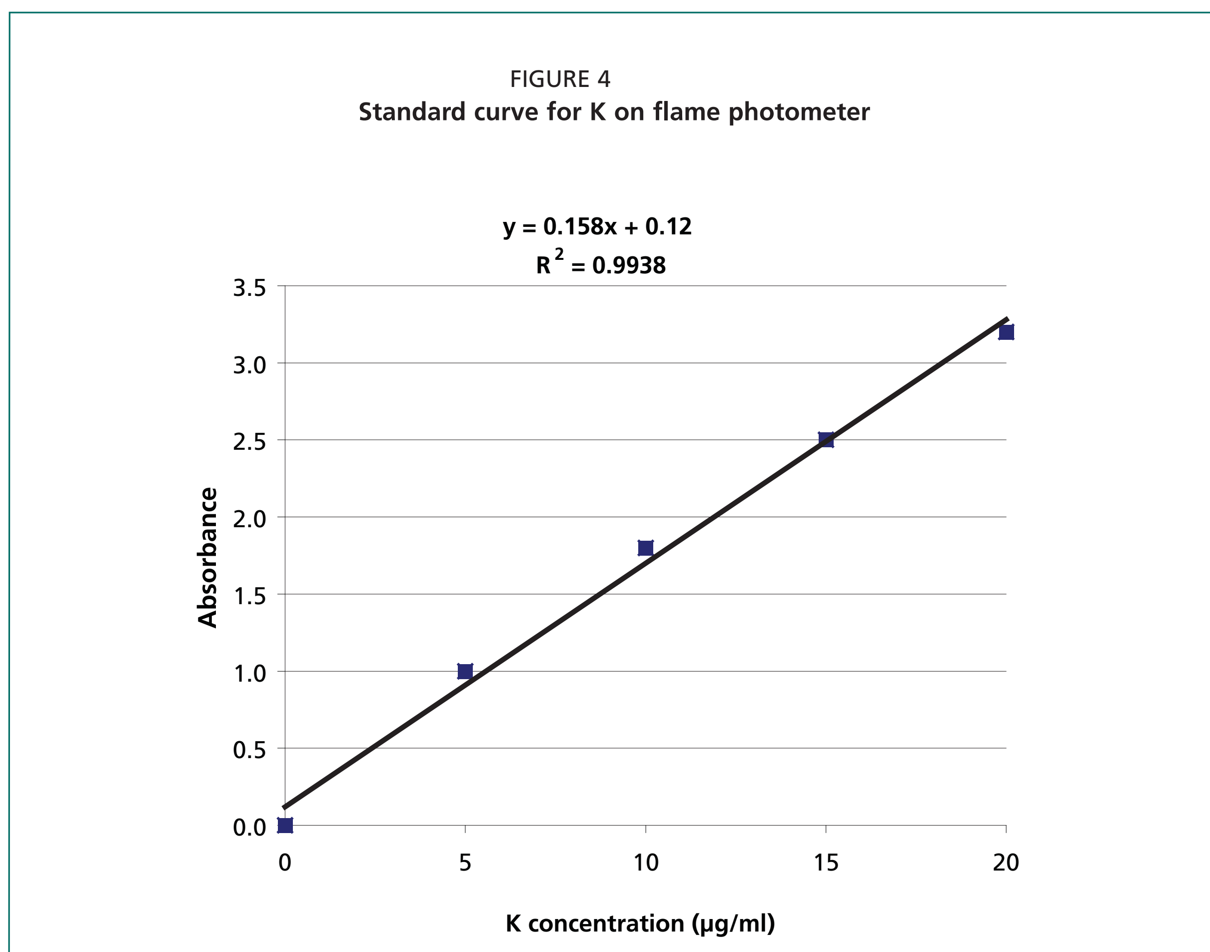
$$K\ (kg/ha) = \frac{A}{1\,000\,000} \times 25 \times \frac{2\,000\,000}{5}$$

where:

- A = content of K (µg) in the sample, as read from the standard curve;
- volume of the extract = 25 ml;
- weight of the soil taken = 5 g;
- weight of 1 ha of soil down to a plough depth of 22 cm is taken as 2 million kg.

Figure 4 shows an example of a standard curve for estimating K using the flame photometer method.

FIGURE 4
Standard curve for K on flame photometer



Available sulphur

Available S in mineral soils occurs mainly as adsorbed SO_4 ions. Phosphate ions (as monocalcium phosphate) are generally preferred for replacement of the adsorbed SO_4 ions. The extraction is also carried out using $CaCl_2$ solution. However, the former is considered to be better for more efficient replacement of SO_4 ions. The use of Ca salts has a distinct advantage over those of Na or K as Ca prevents deflocculation in heavy-textured soils and leads to easy filtration. The SO_4 in the extract can be estimated turbidimetrically using a spectrophotometer. A major problem arises when the amount of S extracted is too low to be measured. To overcome this problem, a seed solution of known S concentration is added to the extract in order to raise the concentration to a readily detectable level.

The apparatus required for the barium sulphate precipitation method of estimating available S (adapted from Singh, Chhonkar and Pandey, 1999) consists of:

- a spectrophotometer;
- a mechanical shaker;
- a volumetric flask.

The reagents required are:

- Monocalcium phosphate extracting solution (500 mg P/litre): Dissolve 2.035 g of $Ca(H_2PO_4)_2.H_2O$ in 1 litre of water.
- Gum acacia – acetic acid solution: Dissolve 5 g of chemically pure gum acacia powder in 500 ml of hot water and filter in hot condition through No. 42 filter paper. Cool and dilute to 1 litre with dilute acetic acid.
- Barium chloride: Pass AR-grade $BaCl_2$ salt through a 1-mm sieve and store for use.
- Standard stock solution (2 000 mg S/litre): Dissolve 10.89 g of oven-dried AR-grade potassium sulphate in 1 litre of water.
- Standard working solution (10 mg S/litre): Measure exactly 2.5 ml of the stock solution and dilute to 500 ml.
- Barium sulphate seed suspension: Dissolve 18 g of AR-grade $BaCl_2$ in 44 ml of hot water and add 0.5 ml of the standard stock solution. Heat the content to boiling and then cool quickly. Add 4 ml of gum acacia – acetic acid solution to it. Prepare a fresh seed suspension for estimation every day.
- Dilute nitric acid (about 25 percent): Dilute 250 ml of AR-grade concentrated HNO_3 to 1 litre.
- Acetic-phosphoric acid: Mix 900 ml of AR-grade glacial acetic acid with 300 ml of H_3PO_4 (AR-grade).

The procedure is:

1. Weigh 20 g of soil sample in a 250-ml conical flask. Add 100 ml of the monocalcium phosphate extracting solution (500 mg P/litre) and shake for 1 hour. Filter through No. 42 filter paper.
2. Put 10 ml of the clear filtrate in a 25-ml volumetric flask.
3. Add 2.5 ml of 25 percent HNO_3 and 2 ml of acetic-phosphoric acid. Dilute to about 22 ml, stopper the flask and shake well (if required).

4. Shake the $BaSO_4$ seed suspension and then add 0.5 ml of it, and 0.2 g of $BaCl_2$ crystals. Stopper the flask, invert 3 times, and keep.
5. After 10 minutes, invert 10 times. After another 5 minutes, invert 5 times.
6. Allow to stand for 15 minutes and then add 1 ml of gum acacia-acetic acid solution.
7. Make the volume up to 25 ml, invert 3 times and set aside for 90 minutes.
8. Invert 10 times and measure the turbidity intensity at 440 nm (blue filter).
9. Run a blank side by side.
10. Preparation of standard curve:
    - Put 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 ml of the working standard solution (10 mg S/litre) into a series of 25-ml volumetric flasks in order to obtain 25, 50, 75, 100, 125 and 150 µg of S.
    - Proceed to develop turbidity as described above for sample aliquots.
    - Read the turbidity intensity and prepare the curve by plotting readings against S concentrations (in micrograms in the final volume of 25 ml).

The calculation is:

$$\text{Available sulphur } (SO_4^- - S) \text{ in soil (mg/kg)} = \frac{W \times 100}{10 \times 20} = \frac{W}{2}$$

where:
- *W* stands for the quantity of S (in milligrams) as obtained on the x-axis against an absorbance reading (y-axis) on the standard curve;
- 20 is the weight of the soil sample (in grams);
- 100 is the volume of the extractant (in millilitres);
- 10 is the volume of extractant (in millilitres) in which turbidity is developed.

Exchangeable calcium and magnesium

Exchangeable cations are usually determined in a neutral normal ammonium acetate extract of soil. Extraction is carried out by shaking the soil–extractant mixture, followed by filtration or centrifugation. Calcium and Mg are determined either by the EDTA titration method or by using an AAS after the removal of ammonium acetate and OM.

Appreciable amounts of soluble Calcium and Mg may be present is soils. Hence, these water-soluble cations are estimated in the 1:2 soil–water extract and deducted from ammonium acetate extractable Ca and Mg (as ammonium acetate also extracts water-soluble cations) in order to obtain exchangeable Ca and Mg. Generally, to obtain the soil–water extract, 25 g soil and 50 ml of water suspension is shaken for 30 minutes on a mechanical shaker and filtered. The method of estimation in the water extract (water-soluble cations) and ammonium acetate extract (exchangeable cations) is same.

The EDTA titration method developed by Cheng and Bray (1951) is preferred on account of its accuracy, simplicity and speed.

The method is based on the principle that Ca, Mg and a number of other cations form stable complexes with versenate (EDTA disodium salt) at different pH values. The interference of Cu, Zn, Fe and Mn is prevented by the use of 2 percent NaCN solution or carbamate. Usually, in irrigation waters and water extracts of soil, the quantities of interfering ions are negligible and can be ignored.

A known volume of a standard Ca solution is titrated with standard versenate 0.01N solution using muroxide (ammonium purpurate) indicator in the presence of NaOH solution. The end point is a change in colour from orange-red to purple at pH 12 when all the Ca forms a complex with EDTA.

Calcium by the versenate (EDTA) method

The apparatus required consists of:

- a shaker;
- a porcelain dish;
- some beakers;
- a volumetric/conical flask.

The reagents required are:

- Ammonium chloride – ammonium hydroxide buffer solution: Dissolve 67.5 g of ammonium chloride in 570 ml of concentrated ammonium hydroxide, and make up to 1 litre.
- Standard 0.01N Ca solution: Take accurately 0.5 g of pure calcium carbonate and dissolve it in 10 ml of 3N HCl. Boil to expel CO_2 and then make the volume up to 1 litre with distilled water.
- EDTA solution (0.01N): Take 2.0 g of versenate, dissolve in distilled water and make the volume up to 1 litre. Titrate it with 0.01N Ca solution and make the necessary dilution so that its normality is exactly equal to 0.01N.
- Muroxide indicator powder: Take 0.2 g of muroxide and mix it with 40 g of powdered potassium sulphate. This indicator should not be stored in the form of solution, otherwise it oxidizes.
- Sodium diethyl dithiocarbamate crystals: These are used to remove interference by other metal ions.
- Sodium hydroxide 4N: Prepare 16 percent soda solution by dissolving 160 g of pure sodium hydroxide in water, and make the volume up to 1 litre. This will give pH 12.

The procedure is:

1. Put 5 g of air-dried soil sample in a 150-ml conical flask and add 25 ml of neutral normal ammonium acetate. Shake on a mechanical shaker for 5 minutes and filter through No. 1 filter paper.
2. Take a suitable aliquot (5 or 10 ml) and add 2–3 crystals of carbamate and 5 ml of 16 percent NaOH solution.
3. Add 40–50 mg of the indicator powder. Titrate it with 0.01N EDTA solution until the colour changes gradually from orange-red to reddish-violet (purple). Add a drop of EDTA solution at intervals of 5–10 seconds, as the change of colour is not instantaneous.

4. The end point must be compared with a blank reading. If the solution is overtitrated, it should be backtitrated with standard Ca solution; thus, the exact volume used is found.
5. Note the volume of EDTA used for titration.

The calculation is:

If N_1 is normality of Ca^{2+} and V_1 is volume of aliquot taken and N_2V_2 are the normality and volume of EDTA used, respectively, then:

$$N_1V_1 = N_2V_2$$

$$N_1 = \frac{N_2V_2}{V_1} = \frac{\text{Normality of EDTA x Vol. of EDTA}}{\text{ml of aliquot taken}}$$

Here, N_1 (normality) = equivalent of Ca^{2+} present in 1 litre of aliquot. Hence, Ca^{2+} me/litre is:

$$\frac{\text{Normality of EDTA x Vol. of EDTA x 1000}}{\text{ml of aliquot taken}}$$

When expressed on soil weight basis, Ca^{2+} me/100 g soil is:

$$\frac{100}{\text{wt. of soil}} \text{ x } \frac{\text{extract volume}}{1000} \text{ x Ca as me/litre}$$

Calcium plus magnesium by the versenate (EDTA) method

Magnesium in solution can be titrated with 0.01N EDTA using EBT dye as indicator at pH 10 in the presence of ammonium chloride and ammonium hydroxide buffer. At the end point, the colour changes from wine-red to blue or green. Where Ca is also present in the solution, this titration will estimate both Ca and Mg. Beyond pH 10, Mg is not bound strongly to EBT indicator to give a distinct end point.

The apparatus required consists of:

- a shaker;
- a porcelain dish;
- a beaker;
- a volumetric/conical flask.

The reagents required are:

- EDTA or versenate solution (0.01N): Same as for Ca determination (above).
- Ammonium chloride – ammonium hydroxide buffer solution: Same as for Ca determination (above).
- EBT indicator: Take 100 ml of ethanol, and dissolve 4.5 g of hydroxylamine hydrochloride in it. Add 0.5 g of the indicator and prepare the solution. Hydroxylamine hydrochloride removes the interference of Mn by keeping it in a lower valency state (Mn^{2+}).

- Sodium cyanide solution (2 percent) or sodium diethyl dithiocarbamate crystals: This is used to remove the interference of Cu, Co and nickel (Ni).

The procedure is:

1. Put 5 g of air-dried soil in a 150-ml flask, add 25 ml of neutral normal ammonium acetate solution, shake on a mechanical shaker for 5 minutes, and filter through No. 1 filter paper.
2. Pipette out 5 ml of aliquot containing not more than 0.1 me of Ca plus Mg. If the solution has a higher concentration, it should be diluted.
3. Add 2–5 crystals of carbamate and 5 ml of ammonium chloride – ammonium hydroxide buffer solution. Add 3–4 drops of EBT indicator.
4. Titrate this solution with 0.01N versenate until the colour changes to bright blue or green and no tinge of wine-red colour remains.

For the calculation, if N_1 and V_1 are normality (concentration of $Ca^{2+} + Mg^{2+}$) and volume of aliquot taken, and N_2V_2 are the normality and volume of EDTA used, respectively, then, $N_1V_1 = N_2V_2$; or $N_1 =$

$$\frac{N_2V_2}{V_1} = \frac{\text{Normality of EDTA x Vol. of EDTA}}{\text{ml of aliquot taken}}$$

Here, N_1 (normality) = equivalents of Ca^{2+} plus Mg^{2+} present in 1 litre of aliquot. Hence:

$$Ca^{2+} \text{plus } Mg^{2+} \text{ me/litre} = \frac{\text{Normality of EDTA x Vol. of EDTA x 1000}}{\text{ml of aliquot taken}}$$

Milli-equivalent (me) of Mg^{2+} = me ($Ca^{2+} + Mg^{2+}$) – me of Ca^{2+}

When expressed on a soil weight basis, $Ca^{2+} + Mg^{2+}$ me/100 g soil =

$$\frac{100}{\text{wt. of soil}} \text{ x } \frac{\text{extract volume}}{1000} \text{ x } Ca^{++} + Mg^{++} \text{ me/litre}$$

Micronutrients

For the estimation of micronutrients in soils, it is the plant-available form that is critical and not the total content. The major objective of soil testing for micronutrients, as with macronutrients, is to determine whether a soil can supply adequate micronutrients for optimal crop production or whether nutrient deficiencies are expected in crops grown on such soils. The most commonly studied micronutrients are Zn, Cu, Fe, Mn, B and Mo, and the same are dealt with here.

Micronutrients are present in different forms in the soil. Among the most deficient ones is Zn, which is present as the divalent cation Zn^{2+}. Maize, citrus, legumes, cotton and rice are especially sensitive to Zn deficiency. Iron is present mostly in sparingly soluble ferric oxide forms, which occur as coatings of aggregate or as separate constituents of the clay fraction. Soil redox potential and

pH affect Fe availability. The Fe form that is predominantly taken up by plants is Fe^{2+}. The uptake of Fe is inhibited by phosphate levels caused by the formation of insoluble iron phosphate. Chemically, Mn behaves in soil in the same way as Fe. Soil Mn originates primarily from the decomposition of ferromagnesian rocks. It is taken up by the plants as Mn^{2+} ions although it exists in many oxidation states. Manganese and phosphate are mutually antagonistic. Copper, as zinc, exists in soils mainly as divalent ions, Cu^{2+}. It is usually adsorbed by clay minerals or associated with OM although they have little or no effect on its availability to crops. High phosphate fertilization can induce Cu deficiency. Molybdenum usually occurs as MoO_3, MoO_5 and MoO_2. These oxides are transformed slowly to soluble molybdates (MoO_4), which is the form taken up by plants. Boron deficiency occurs mostly in the light-textured acid soils when they are leached heavily through irrigation or heavy rainfall.

There are different extractants for assessing plant-available nutrient (element) content in soils. The elements so extracted can be estimated quantitatively through chemical methods or instrumental techniques. Table 10 lists commonly used extractants for different elements.

Zinc, copper, iron and manganese

Ethylenediamine tetraacetic acid (EDTA) with ammonium acetate is commonly used for the extraction of many elements. Diethylenetriamine pentaacetic acid (DTPA) is another common (universal) extractant and it is widely used for the simultaneous extraction of elements such as Zn, Cu, Fe and Mn (Lindsay and Norvell, 1978). Although a specific extractant for an element that has a higher correlation with plant availability may be preferred, the universal or common extractant saves on the cost of chemicals and the time involved in estimation, especially in a service laboratory where a large number of samples need to be analysed in a short period.

The estimation of elements in the extract is done with the help of an AAS. Table 11 details the critical limits for DTPA-extractable micronutrient elements as proposed by Lindsay and Norvell (1978).

Diethylenetriamine pentaacetic acid is an important and widely used chelating agent that combines with free metal ions in the solution to form soluble complexes

TABLE 10
Commonly used extractants for micronutrients

Element	Extractants
Zinc	EDTA + ammonium acetate, EDTA + ammonium carbonate, DTPA + $CaCl_2$, HCl, HNO_3 and dithiozone + ammonium acetate
Copper	EDTA, EDTA + ammonium acetate, ammonium bicarbonate + DTPA, HCl and HNO_3
Iron	EDTA, DTPA, EDTA + ammonium acetate, HCl and HNO_3
Manganese	Hydroquinone, ammonium phosphate, DTPA and EDTA + ammonium acetate
Boron	Hot water and mannitol + $CaCl_2$
Molybdenum	Ammonium oxalate, ammonium acetate, ammonium fluoride and water

TABLE 11
Critical limits for DTPA-extractable micronutrients

Availability	Micronutrients			
	Zn	Cu	Fe	Mn
		(μg/g soil)		
Very low	0–0.5	0–0.1	0–2	0–0.5
Low	0.5–1	0.1–0.3	2–4	0.5–1.2
Medium	1–3	0.3–0.8	4–6	1.2–3.5
High	3–5	0.8–3	6–10	3.5–6
Very high	> 5	> 3	> 10	> 6

of elements. To avoid excessive dissolution of CaCO3, which may release occluded micronutrients that are not available to crops in calcareous soils and may give erroneous results, the extractant is buffered in slightly alkaline pH. Triethanolamine (TEA) is used as buffer because it burns cleanly during atomization of extractant solution while estimating on an AAS. The DTPA has a capacity to complex each of the micronutrient cations as 10 times of its atomic weight. The capacity ranges from 550 to 650 mg/kg depending on the micronutrient cations.

To prepare DTPA 0.005M, 0.01M $CaCl_2.2H_2O$ and 0.1M TEA extractant:

1. Add 1.967 g of DTPA and 13.3 ml of TEA in 400 ml of distilled water in a 500-ml flask.
2. Put 1.47 g of $CaCl_2.2H_2O$ in a separate 1 000-ml flask. Add 500 ml of distilled water and shake to dissolve.
3. Add DTPA+TEA mixture to the $CaCl_2$ solution and make the volume up to 1 litre. Adjust the pH to 7.3 by using 1M HCl before making the volume.

The extracted elements can be estimated by various methods, including volumetric analysis, spectrometry and atomic absorption spectroscopy. Volumetric methods such as EDTA and $KMnO_4$ titrations are used for estimating Zn and Mn, and Fe, respectively. Copper can be estimated by titration with $Na_2S_2O_3$. Spectrometric methods are used in the estimation of a specific colour developed because of the presence of an element that forms coloured compounds in the presence of specific chemicals under a definite set of conditions. The colour intensity has to be linear with the concentration of the element in question. The interference caused by any other element has to be eliminated. Such methods are:

- the dithiozone method for Zn;
- the orthophenonthroline method for Fe;
- the potassium periodate method for Mn;
- the carbamate method for Cu.

These methods are generally cumbersome and time-consuming. Therefore, the most commonly employed method is atomic absorption spectrometry. Here, the interference by other elements is almost nil or negligible because the estimation is carried out for an element at a specific emission spectraline. In fact, in atomic absorption spectrometry, traces of one element can be determined accurately in the presence of a high concentration of other elements.

The procedure is based on flame absorption rather than flame emission and on the fact that metal atoms absorb strongly at discrete characteristic wavelengths that coincide with the emission spectralines of a particular element. The liquid sample is atomized. A hollow cathode lamp (which precedes the atomizer) emits the spectrum of the metal used to make the cathode. This beam traverses the flame and is focused on the entrance slit of a monochromator, which is set to read the intensity of the chosen spectraline. Light with this wavelength is absorbed by the metal in the flame, and the degree of absorption being the function of the concentration of the metal in the flame, the concentration of the atoms in the dissolved material is determined. For elemental analysis, a working curve or a standard curve is prepared by measuring the signal or absorbance of a series of standards of known concentration of the element under estimation. From such a curve, the concentration of the element in the unknown sample is estimated.

Atomic absorption spectroscopy can be applied successfully for estimating Zn, Cu, Fe and Mn. For specific estimation with an AAS, hollow cathode lamps specific to specific elements are used. Table 12 lists the relevant parameters.

The software provided with the equipment manual details the operating parameters that are specific to a particular model. Accordingly, the current supply, wavelength of hollow cathode lamp, integration time and anticipated estimation ranges are fixed. Hollow cathode and deuterium lamps need to be aligned properly before starting the equipment. After proper alignment and adjustment, standard curves are prepared to ensure that the concentration of the elements in solutions relates perfectly to the absorbance.

Ready-made standard solutions of 1 000 µg/ml or 1 mg/ml (1 000 ppm) of dependable accuracy are supplied with the AAS and are also available from suppliers of chemical reagents. Where the standard solutions are to be prepared in the laboratory, either metal element foils of 100-percent purity or the standard chemical salts can be used. Table 13 details the quantities of chemical required to make 1 litre of standard solution of 100 µg/ml for different elements.

In the case of Zn, Cu and Fe, 1 000 µg/ml (1 000 ppm) standard solutions are preferably prepared by dissolving 1.0 g of pure metal wire and making the volume up to 1 litre as per the method described under each element. The solution is diluted to obtain the required concentration. In the case of Mn, $MnSO_4.H_2O$ is preferred.

TABLE 12
Parameters for estimation of micronutrients using an AAS

Specifications	Zn	Cu	Fe	Mn
Lamp current (m A°)	5	3	7	5
Wavelength (nm)	213.9	324.8	248.3	279.5
Linear range (mg/litre)	0.4–1.5	1.0–5.0	2.0–9.0	1.0–3.6
Slit width (nm)	0.5	0.52	0.2	0.2
Integration time (seconds)	2.0	2.0	2.0	2.0
Flame	Air acetylene			

TABLE 13
Specifications for preparing micronutrient standard solutions

Element	Concentration of stock solution	Salt to be used	Quantity of salt required
	(µg/ml)		*(g/litre)*
Zn	100	Zinc sulphate ($ZnSO_4.7H_2O$)	0.4398
Cu	100	Copper sulphate ($CuSO_4.5H_2O$)	0.3928
Fe	100	Ferrous sulphate ($FeSO_4.7H_2O$) or	0.4964
		Ferrous ammonium sulphate	0.7028
Mn	100	Manganese sulphate ($MnSO_4.H_2O$)	0.3075

Preparation of standard curve for zinc

The reagents required are:

- Standard Zn solution: Weigh 1.0 g of pure zinc metal in a beaker. Add 20 ml of HCl (1:1). Keep for a few hours, allowing the metal to dissolve completely. Transfer the solution to a 1-litre volumetric flask. Make up the volume with glass-distilled water. This is 1 000 µg/ml Zn solution. For preparing the standard curve, refer to the 1 000 µg/ml solution as solution A. Dilute 1 ml of standard A to 100 ml in order to obtain a 10 µg/ml solution, to be designated standard B.
- Glass-distilled or demineralized acidified water of pH 2.5 ± 0.5: Dilute 1 ml of 10 percent sulphuric acid to 1 litre with glass-distilled or mineralized water and adjust the pH to 2.5 with a pH meter using 10 percent H_2SO_4 or NaOH. This solution is called acidified water.
- Working Zn standard solutions: Pipette 1, 2, 4, 6, 8 and 10 ml of standard B solution in 50-ml numbered volumetric flasks and make the volume up with DTPA solution to obtain 0.2, 0.4, 0.8, 1.2, 1.6 and 2.0 µg/ml zinc. Stopper the flasks and shake them well. Fresh standards should be prepared every time when a fresh lot of acidified water is prepared.

The procedure is:

1. Flaming the solutions: Atomize the standards on an AAS at a wavelength of 213.8 nm (Zn line of the instrument).
2. Prepare a standard curve of known concentrations of Zn solution by plotting the absorbance values on the y-axis against their respective Zn concentration on the x-axis.

Special points to note are:

- Weighing must be done on an electronic balance.
- All the glass apparatus to be used should be washed first with dilute hydrochloric acid (1:4) and then with distilled water.
- The pipette should be rinsed with the same solution to be measured.
- The outer surface of the pipette should be wiped with filter paper after use.

- After using the pipette, place it on a clean dry filter paper in order to prevent contamination.

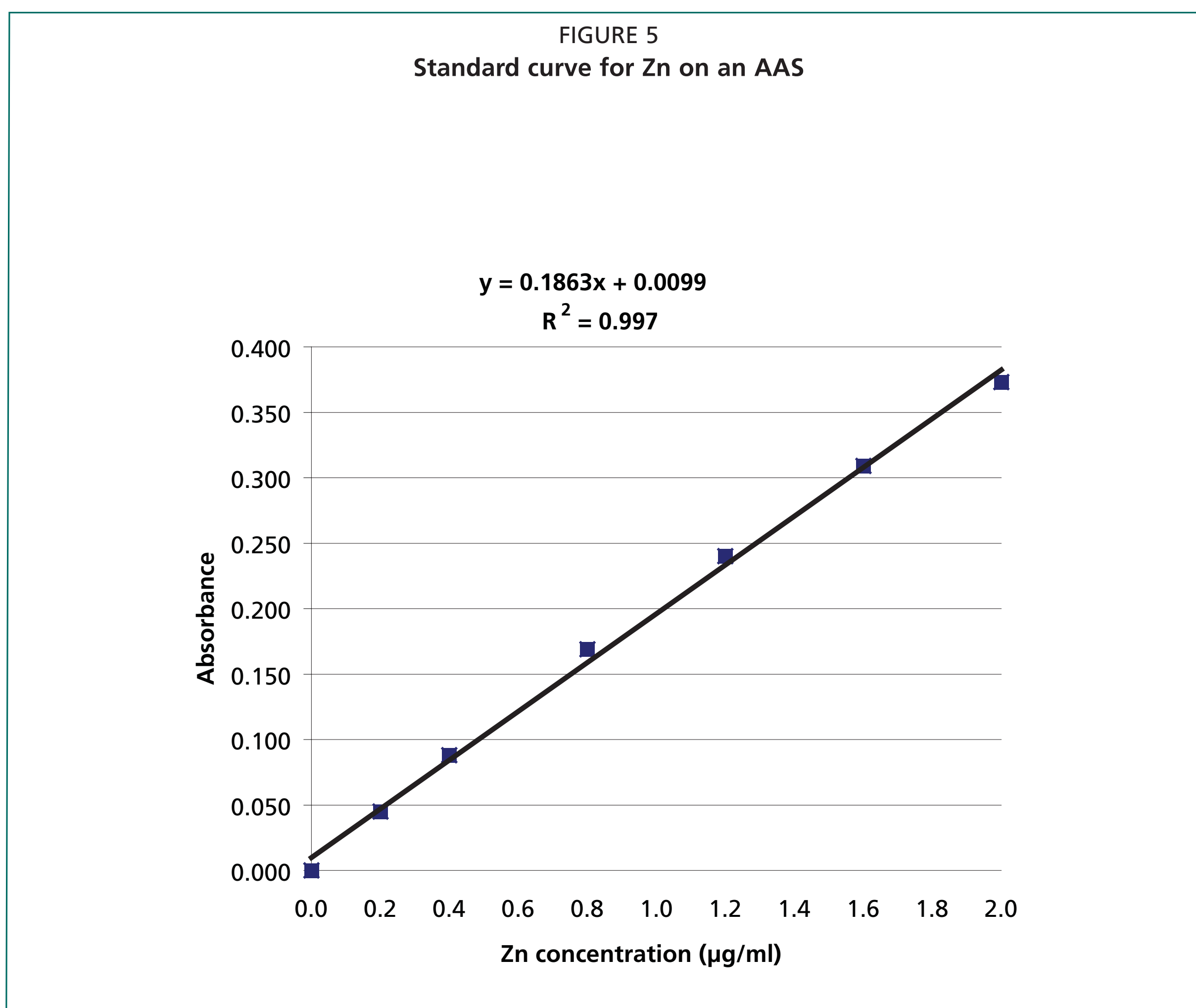
As an example, Figure 5 shows a standard curve prepared by the authors for Zn estimation while establishing a soil testing laboratory.

Preparation of standard curve for copper

The reagents required are:

- Standard Cu solution: Weigh 1 g of pure copper wire on a clean watch glass and transfer it to a 1-litre flask. Add 30 ml of HNO_3 (1:1) and make up to the mark by glass-distilled water. Stopper the flask and shake the solution well. This is 1 000 µg/ml Cu solution and it should be stored in a clean bottle for further use. Dilute 1 ml of 1 000 µg/ml solution of Cu to 100 ml to obtain 10 µg/ml of standard Cu solution.
- Glass-distilled or demineralized acidified water of pH 2.5 ± 0.5: Same as that for Zn (above).

FIGURE 5
Standard curve for Zn on an AAS



➢ Working Cu standard solutions: Pipette 2, 3, 4, 5, 6 and 7 ml of 10 µg/ml of standard Cu solution in 50-ml numbered volumetric flasks and make the volume up with DTPA solution to obtain 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 µg/ml Cu. Stopper the flasks and shake them well. Prepare fresh standards every fortnight.

The procedure is:

1. Flame the standards on an AAS at a wavelength of 324.8 nm (Cu line of the instrument).
2. Prepare the standard curve with the known concentration of Cu on the x-axis by plotting against the absorbance value on the y-axis.

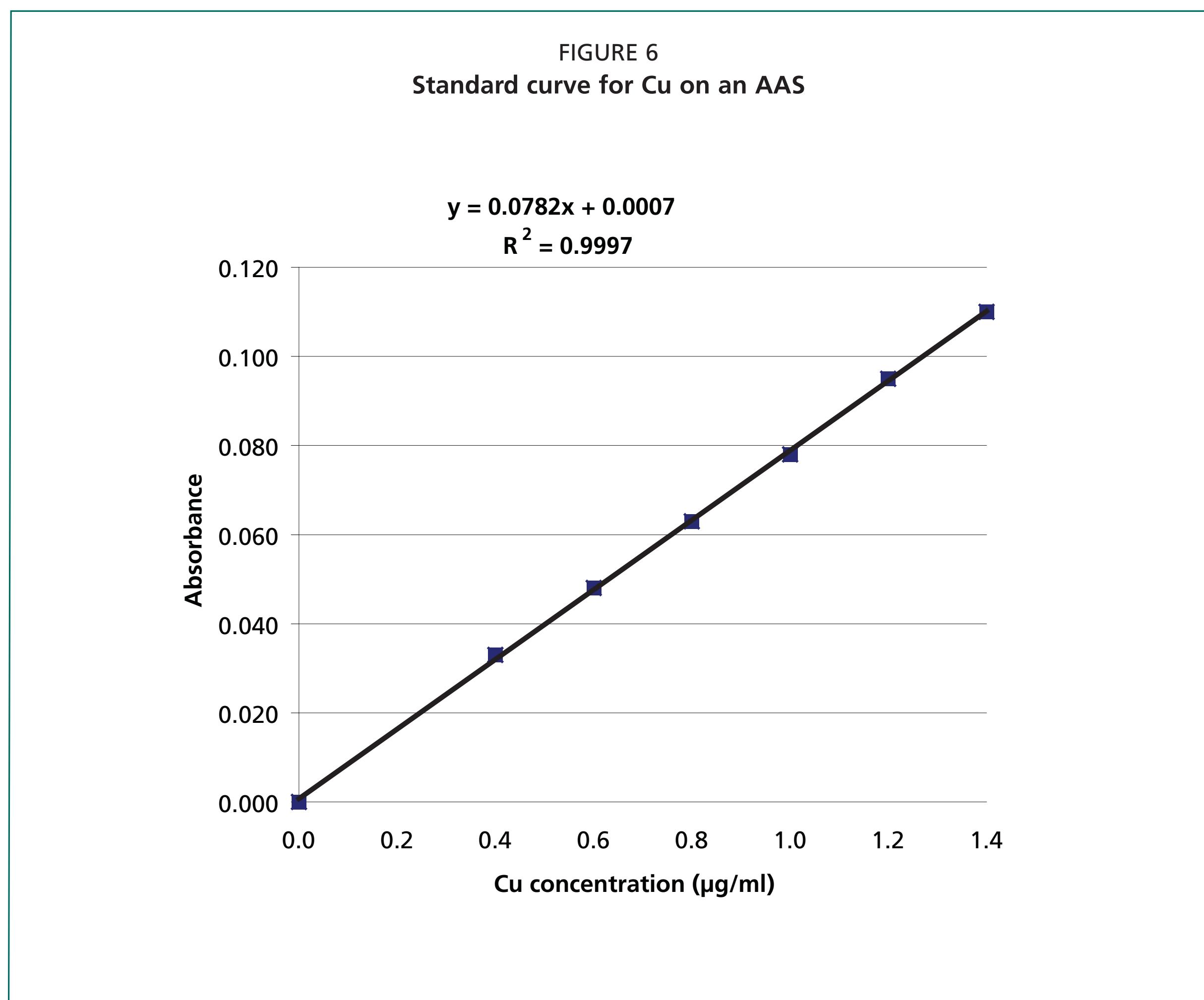
As an example, Figure 6 shows a standard curve prepared by the authors for Cu estimation while establishing a soil testing laboratory.

Preparation of standard curve for iron

The reagents required are:

➢ Standard Fe solution: Weigh accurately 1 g of pure iron wire, put it in a beaker, and add about 30 ml of 6M HCl and boil. Transfer it to a 1 litre

FIGURE 6
Standard curve for Cu on an AAS



volumetric flask through a funnel, giving several washings to the beaker and funnel with glass-distilled water. Make the volume up to the mark. Stopper the flask and shake the solution well. This is 1 000 µg/ml iron solution.

- Glass-distilled or demineralized acidified water of pH 2.5 ± 0.5: Same as that for Zn (above).
- Working Fe standard solutions: Pipette 10 ml of Fe stock solution in a 100-ml volumetric flask, and dilute to volume with DTPA solution. This is 100 µg/ml iron solution. Take 2, 4, 8, 12 and 16 ml of 100 µg/ml solution and dilute each to 100 ml to obtain 2, 3, 8, 12 and 16 µg/ml of Fe solution.

The procedure is:

1. Flame the standards on an AAS at a wavelength of 248.3 nm (Fe line of the instrument).
2. Prepare the standard curve with the known concentration of Cu on x-axis by plotting against the absorbance value on the y-axis.

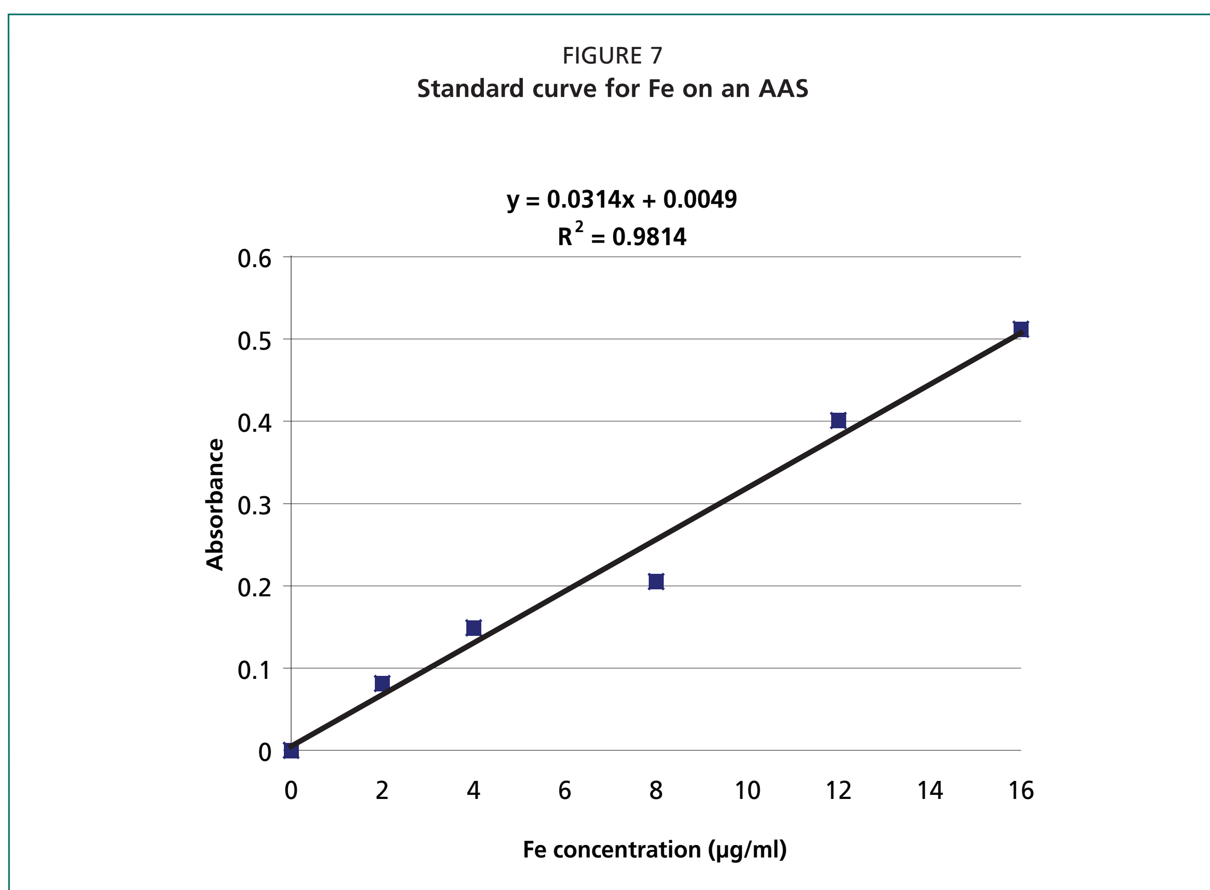
As an example, Figure 7 shows a standard curve prepared by the authors for Fe estimation while establishing a soil testing laboratory.

Preparation of standard curve for manganese

The reagents required are:

- Standard Mn solution: Weigh 3.0751 g of AR-grade manganese sulphate ($MnSO_4.H_2O$) on a clean watch glass and transfer it to a 1-litre flask through

FIGURE 7
Standard curve for Fe on an AAS



a funnel, giving several washings to the watch glass and funnel with acidified water, and make the volume up to the mark. This solution will be 1 000 µg/ml Mn. A secondary dilution of 5 ml to 100 ml with acidified water gives a 50 µg/ml solution.

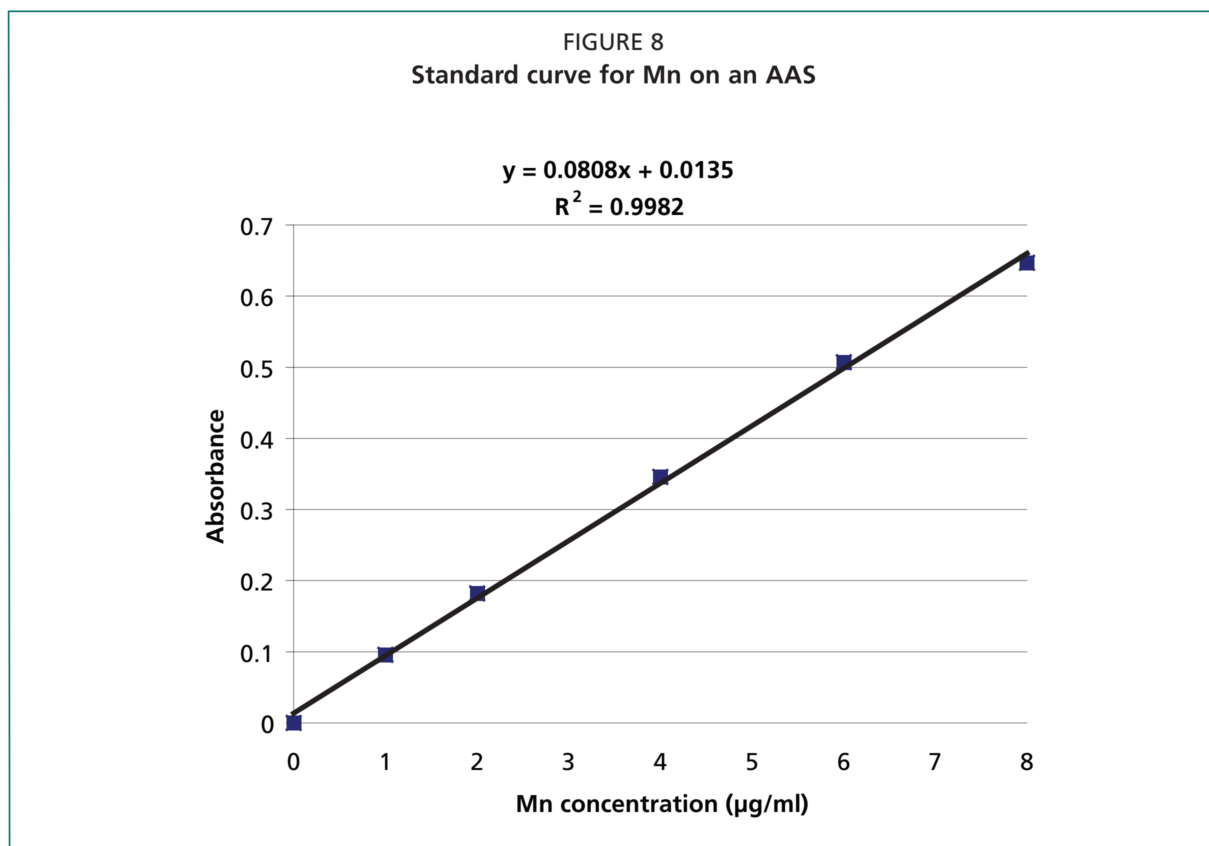
- Glass-distilled or demineralized acidified water of pH 2.5 ± 0.2: Same as that for Zn (above).
- Working Mn standard solutions: The standard curve is prepared by taking lower concentrations of Mn in the range of 0–10 µg/ml. Take 1, 2, 4, 6 and 8 ml of 50 µg/ml solution, and make the volume up with DTPA solution to 50 ml to obtain 1, 2, 4, 6 and 8 µg/ml working standards.

The procedure is:

1. Flame the standards on an AAS at a wavelength of 279.5 nm (Mn line of the instrument).
2. Prepare the standard curve with the known concentration of Mn on the x-axis by plotting against the absorbance value on the y-axis.

As an example, Figure 8 shows a standard curve prepared by the authors for Mn estimation while establishing a soil testing laboratory.

FIGURE 8
Standard curve for Mn on an AAS



Procedure for extraction by DTPA

Once standard curves have been prepared, proceed for extraction by DTPA as follows:

1. Put 10 g of the soil sample in a 100-ml narrow-mouthed polypropylene bottle.
2. Add 20 ml of DTPA extracting solution.
3. Stopper the bottle, and shake for 2 hours at room temperature (25 °C).
4. Filter the content using filter paper No. 1 or No. 42, and collect the filtrate in polypropylene bottles.
5. Prepare a blank following all steps except taking a soil sample.

The extract so obtained is used for estimation of different micronutrients. For extraction of a more accurate quantity of an element that has a higher degree of correlation with plant availability, there are element-specific extractants. An extractant standardized/recommended for a given situation in a country may be used. However, the estimation procedure on an AAS remains unchanged.

Estimation on an AAS

The procedure is:

1. Select an element-specific hollow cathode lamp and mount it on the AAS.
2. Start the flame.
3. Set the instrument at zero by using blank solution.
4. Aspirate the standard solutions of different concentrations one by one and record the readings.
5. Prepare the standard curve, plotting the concentration of the element concerned and the corresponding absorbance in different standard samples (as described above).
6. When the operation is performed accurately, a straight line relationship is obtained between the concentration of the element and the absorbance on the AAS with a correlation coefficient that may be nearly as high as 1.0.
7. Aspirate the soil extractant obtained for estimation of the nutrient element in the given soil sample and observe the readings.
8. Determine the content of the element in the soil extract by observing its concentration on the standard curve against its absorbance

The relevant calculation is:

Content of micronutrient in the sample (mg/kg) = C µg/ml × 2 (dilution factor)

where:

- dilution factor = 2.0 (soil sample taken = 10.0 g and DTPA used = 20 ml);
- absorbance reading on AAS of the soil extract being estimated for a particular element = X
- concentration of micronutrient as read from the standard curve for the given absorbance (X) = C µg/ml.

Available boron

The most commonly used method for available B is hot water extraction of soil as developed by Berger and Truog (1939). A number of modified versions of this method have been proposed but the basic procedure remains the same.

Water-soluble B is the available form of B. It is extracted from the soil by water suspension. In the extract, B can be analysed by colorimetric methods using reagents such as carmine, azomethine-H, and, most recently, by inductively coupled plasma (ICP) and atomic emission spectrometry. However, the colorimetric method is preferable owing to the fact that as B is a non-metal, the use of an AAS for its estimation poses some limitations.

The extraction procedure for the methods presented below is:

- Put 25 g of soil in a quartz flask or beaker.
- Add about 50 ml of double-distilled water (DDW) and about 0.5 g of activated charcoal.
- Boil the mixture for about 5 minutes, and filter through No. 42 filter paper.

Estimation by AAS

The specifications/relevant parameters for estimation of B on an AAS are:

- lamp current: 20 m A°;
- wavelength: 249.7 nm;
- linear range: 1–10 µg/ml;
- slit width: 0.2 nm;
- integration time: 2.0 seconds;
- flame: acetylene nitrous oxide.

The software provided with the equipment manual gives the operating parameters that are specific to a particular model. Accordingly, the current supply, wavelength of hollow cathode lamp, integration time and anticipated estimation ranges are fixed. Hollow cathode and deuterium lamps need to be aligned properly before starting the equipment. After proper alignment and adjustment, standard curves are prepared in order to ensure that the concentration of the element in solutions relates perfectly to the absorbance.

The reagents required are:

- Standard B solution: Dissolve 8.819 g of $Na_2B_4O_7.10H_2O$ in warm water. Dilute to 1 litre to obtain 1 000 µg/ml B stock solution. Dilute 1 ml of standard to 100 ml to obtain 10 µg/ml B.
- Working standards: Take 1, 2, 3, 4, 5, 6, 7 and 10 ml of 10 µg/ml solution and dilute each to 50 ml to obtain 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 2.0 µg/ml B.

The procedure is:

1. Atomize the working standards on an AAS using acetylene nitrous oxide as fuel instead of air acetylene fuel (as used for other micronutrients) at a wavelength of 249.7 nm.

2. Prepare a standard curve of known concentration of B by plotting the absorbance values on the y-axis against their respective B concentration on the x-axis. Measure the absorbance of the soil sample extract and determine the B content in the soil from the standard curve.

The relevant calculation is:

Content of B in the sample (mg/kg) = C µg/ml × 2 (dilution factor)

where:

- C = concentration of B in the sample, as read from the standard curve for the given absorbance;
- dilution factor = 2.0 (soil sample taken = 25 g and water used = 50 ml).

Estimation by colorimetric method

The extracted B in the filtered extract is determined by the azomethine-H colorimetric method.

The apparatus required consists of:

- an analytical balance;
- a flask or beaker;
- a volumetric flask;
- some funnels;
- some No. 42 filter paper;
- a spectrophotometer.

The reagents required are:

- Azomethine-H: Dissolve 0.45 g of azomethine-H and 1.0 g of L-ascorbic acid in about 100 ml of deionized or double-distilled water. If the solution is not clear, it should be heated gently in a water-bath or under a hot water tap at about 30 °C until it dissolves. Every week, a fresh solution should be prepared and kept in a refrigerator.
- Buffer solution: Dissolve 250 g of ammonium acetate in 500 ml of deionized or double-distilled water, and adjust the pH to about 5.5 by adding slowly about 100 ml of glacial acetic acid, stirring continuously.
- EDTA solution (0.025 M): Dissolve 9.3 g of EDTA in deionized or double-distilled water, and make the volume up to 1 litre.
- Standard stock solution: Dissolve 0.8819 g of $Na_2B_4O_7.10H_2O$ (AR-grade) in a small volume of deionized water, and make the volume up to 1 000 ml to obtain a stock solution of 100 µg B/ml.
- Working standard solution: Put 5 ml of stock solution in a 100-ml volumetric flask and dilute it to the mark. This solution contains 5 µg B/ml.

The procedure is:

1. Put 5 ml of the clear filtered extract in a 25-ml volumetric flask and add 2 ml of buffer solution, 2 ml of EDTA solution and 2 ml of azomethine-H solution.
2. Mix the contents thoroughly after the addition of each reagent.
3. Let the solution stand for 1 hour to allow colour development. Then, make the volume up to the mark.

4. Measure the intensity of colour at 420 nm. The colour thus developed has been found to be stable for 3–4 hours.
5. Preparation of the standard curve: Put 0, 0.25, 0.50, 1.0, 2.0 and 4.0 ml of 5 µg B/ml solution (working standard) in a series of 25-ml volumetric flasks. Add 2 ml each of buffer reagent, EDTA solution and azomethine-H solution. Mix the contents after each addition and allow to stand at room temperature for 30 minutes. Make the volume up to 25 ml with deionized or double-distilled water, and measure absorbance at 420 nm. This will give reading for standard solution with B concentration of 0, 0.05, 0.10, 0.20, 0.40 and 0.80 µg B/ml.

The relevant calculation is:

Content of B in the soil (µg/g or mg/kg) = C × dilution factor (10)

where:

- C (µg/ml) = concentration of B as read from the standard curve against the absorbance reading of the soil solution on the spectrophotometer;
- dilution factor = 10, which is calculated as follows:
  - weight of the soil taken = 25 g;
  - volume of extractant (water) added = 50 ml;
  - first dilution = 2 times;
  - volume of the filtrate taken = 5 ml;
  - final volume of filtrate after colour development = 25 ml;
  - second dilution = 5 times;
  - total dilution = 2 × 5 = 10 times.

Important points to note are:

- The use of azomethine-H is an improvement over that of carmine, quinalizarin and curcumin because the procedure involving this chemical does not require the use of concentrated acid.
- The amount of charcoal added may vary with the OM content of the soil, and it should be just sufficient to produce a colourless extract after 5 minutes of boiling on a hotplate. Excess amounts of charcoal can result in a loss of extractable B from soils.

Available molybdenum

Molybdenum is a rare element in soils, and it is present only in very small amounts in igneous and sedimentary rocks. The major inorganic source of Mo is molybdenite (MoS_2). The total Mo content in soils is perhaps the lowest of all the micronutrient elements.

In the soil solution, Mo exists mainly as $HMoO_4$ ion under acidic condition, and as MoO_4^{2-} ion under neutral to alkaline conditions. Because of the anionic nature of Mo, its anions will not be attracted much by the negatively charged colloids, and therefore, tend to be leached from the soils in humid region.

Molybdenum can be toxic owing to greater solubility in alkali soils of the arid and semi-arid regions, and deficient in acid soils of the humid regions.

In plants, a deficiency of Mo is common at levels of 0.1 µg/g soil or less. Molybdenum toxicity (molybdenosis) is common where cattle graze forage plants with 10–20 µg Mo/g.

The extraction of Mo usually uses ammonium acetate and/or ammonium oxalate. Estimations can be done both by the AAS and colorimetric methods, with preference for the latter owing to the formation of oxide in the flame in the case of estimation by AAS. Ammonium oxalate is considered a better extractant. However, for estimation on an AAS, ammonium acetate is preferred as the oxalates pose a limitation on the AAS unless removed by digesting with di-acid (below).

Estimation by AAS

The specifications/relevant parameters for estimation of Mo on an AAS are:

- lamp current: 7 m A°;
- wavelength: 313.3 nm;
- linear range: 1–4 µg/ml;
- slit width: 0.2 nm;
- integration time: 2.0 seconds;
- flame: acetylene nitrous oxide.

The software provided with the equipment manual gives the operating parameters that are specific to a particular model. Accordingly, the current supply, wavelength of hollow cathode lamp, integration time and anticipated estimation ranges are fixed. Hollow cathode and deuterium lamps need to be aligned properly before starting the equipment. After proper alignment and adjustment, standard curves are prepared in order to ensure that the concentration of the element in solutions relates perfectly to the absorbance.

The apparatus required consists of:

- a centrifuge and some 50-ml centrifuge tubes;
- an automatic shaker;
- an AAS.

The reagents required are:

- Ammonium acetate solution (NH_4OAc) 1.0M: Dissolve 77.09 g of ammonium acetate in 1 litre of distilled water, and adjust the pH to 7.0.
- Glass-distilled acidified water of pH 2.5: Same as that for Zn estimation (above).
- Standard molybdenum solution: Dissolve 0.15 g of MoO_3 (molybdenum trioxide) in 100 ml of 0.1M NaOH. Dilute to 1 litre to obtain 100 µg/ml Mo stock solution. Dilute 10 ml of the standard to 100 ml to obtain 10 µg/ml Mo.
- Working standard solutions: Take 1, 2, 3, 4, 5, 6, 7 and 10 ml of 10 µg/ml Mo standard solution and dilute each to 50 ml. This will give 0.2, 0.4, 0.6,0.8, 1.0, 1.2, 1.4 and 2.0 µg/ml Mo, respectively.

The procedure is:

1. Weigh accurately 5 g of soil, and transfer it to a 50-ml centrifuge tube.
2. Add 33 ml of 1M ammonium acetate solution to the tube, stopper, and shake in a mechanical shaker for 5 minutes.
3. Centrifuge at 2 000 rpm for 5 minutes or until the supernatant is clear.
4. Decant the solution into a 100-ml volumetric flask.

5. Repeat steps 2–4.
6. Make up the volume to 100 ml with ammonium acetate.
7. Atomize the working standards on an AAS at a wavelength of 313.5 nm. Prepare a standard curve of known concentration of Mo by plotting the absorbance values on the y-axis against their respective Mo concentration on the x-axis.
8. Measure the absorbance of the soil sample extract and determine the Mo content in the soil from the standard curve.

The relevant calculation is:

Content of Mo in the sample (mg/kg) = C µg/ml × 20 (dilution factor).

where:

- C = concentration of Mo in the sample, as read from the standard curve for the given absorbance;
- dilution factor = 20.0 (soil sample taken = 5 g and volume made to 100 ml).

Estimation by colorimetric method

The apparatus required consists of:

- a spectrophotometer;
- a hotplate;
- a refrigerator;
- a water-bath.

The reagents required are:

- 50 percent potassium iodide solution: Dissolve 50 g in 100 ml of DDW.
- 50 percent ascorbic acid solution: Dissolve 50 g in 100 ml of DDW.
- 10 percent sodium hydroxide solution: Dissolve 10 g of NaOH in 100 ml of DDW.
- 10 percent thiourea solution: Dissolve 10 g in 100 ml of DDW and filter. Prepare a fresh solution on the same day of use.
- Toluene-3,4-dithiol solution (commonly called dithiol): Place 1.0 g of AR-grade melted dithiol (51 °C) in a 250-ml glass beaker. Add 100 ml of the 10 percent 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.
- 10 percent tartaric acid: Dissolve 10 g in 100 ml of DDW.
- Iso-amyl acetate.
- Ethyl alcohol.
- Ferrous ammonium sulphate solution: Dissolve 63 g of the salt in about 500 ml of DDW and then make the volume up to 1 litre.
- Nitric–perchloric acid mixture (4:1).
- Extracting reagent: Dissolve 24.9 g of AR-grade ammonium oxalate and 12.6 g of oxalic acid in water, and make the volume up to 1 litre.
- Standard stock solution (100 µg/ml Mo): Dissolve 0.150 g of AR-grade MoO_3 in 100 ml of 0.1M NaOH, make slightly acidic with dilute HCl, and make the volume up to 1 litre.

➢ Working standard solution (1 µg/ml Mo): Dilute 10 ml of the stock solution to 1 litre.

The procedure is:

1. Weigh 25 g of air-dry soil sample in a 500-ml conical flask. Add 250 ml of the extracting solution (1:10 ratio) and shake for 10 hours.
2. Filter through No. 50 filter paper. Collect 200 ml of the clear filtrate in a 250-ml glass beaker and evaporate to dryness in a water-bath.
3. Heat the contents in the beaker at 500 °C in a furnace for 5 hours to destroy OM and oxalates. Keep overnight.
4. Digest the contents with 5 ml of HNO_3–$HClO_4$ mixture (4:1), followed by 10 ml of 4M H_2SO_4 and then with H_2O_2, each time bringing to dryness.
5. Add 10 ml of 0.1M HCl and filter. Wash the filter paper, first with 10 ml of 0.1M HCl and then with 10 ml of DDW, until the volume of the filtrate is 100 ml.
6. Run a blank side by side (without soil).
7. Put 50 ml of the filtrate in 250-ml separatory funnels, add 0.25 ml of ferrous ammonium sulphate solution and 20 ml of DDW, and shake vigorously.
8. Add excess of potassium iodide solution and clear the liberated iodine by adding ascorbic acid drop by drop while shaking vigorously.
9. Add 1 ml of tartaric acid and 2 ml of thiourea solution, and shake vigorously.
10. Add 5 drops of dithiol solution, and allow the mixture to stand for 30 minutes.
11. Add 10 ml of iso-amyl acetate, and separate out the contents (green colour) in colorimeter tubes/cuvettes.
12. Read the colour intensity at 680 nm (red filter).
13. Preparation of standard curve: Measure 0, 2, 5, 10, 15 and 20 ml of the working standard Mo solution containing 1 mg/litre Mo in a series of 250-ml separatory funnels. Proceed for colour development as described above for sample aliquots. Read the colour intensity and prepare the standard curve by plotting Mo concentration against readings.

The relevant calculation is.

Content of Mo in the soil (µg/g or mg/kg) = C µg/ml × dilution factor (0.5)

where:

➢ C (µg/ml) = concentration of Mo as read from the standard curve against the absorbance reading of the soil solution on the spectrophotometer;

➢ dilution factor = 0.5, which is calculated as follows:
- weight of the soil taken = 25 g;
- volume of extract = 250 ml;
- first dilution = 10 times;
- volume of the filtrate taken = 200 ml;
- filtrate digested (concentrated) to 100 ml;
- volume of concentrated filtrate taken = 50 ml;
- second dilution = 0.25 times;

- volume of solvent (iso-amyl acetate) used for extraction = 10 ml;
- third dilution (50 ml extracted by 10 ml) = 0.2 times;
- total dilution = 10 × 0.25 × 0.2 = 0.5 times.

Chapter 4
Plant analysis

Plant and soil testing enables scientific assessment of the needs of the plant for nutrient elements and of the capacity of the soil to supply them. The nutrient elements enter the plant in ionic form from the soil solution. Ion transport to the root surface may take place through ion diffusion and bulk transport (mass flow). Mass flow is the sweeping along of ions as water moves to the root. It is particularly important for ions that are not absorbed on soil colloids such as nitrates and sulphates. Because of mass flow, a plant deficient in N can extract all the nitrates from the soil. Potassium, phosphate and the micronutrient cations are absorbed on soil colloids with various degrees of affinity and are greatly retarded in movement with the soil water. Diffusion (movement along a concentration gradient) is the main mode of transport from the solid phase to the root surface for these non-mobile ions. There is also a possibility that some of them are absorbed by a direct exchange of an ion, usually hydrogen, between the root surface and ions absorbed on soil colloids. Most ions are sorbed by the root against a concentration gradient and, thus, the process involves the use of metabolic energy. The fact that nutrient uptake is an active process explains some of its peculiarities. Plants not only accumulate nutrients against a concentration gradient, they are also able to select from the nutrients at the root surface according to their requirements (preferential uptake).

The understanding of the mechanism of nutrient uptake by plants explains why a chemical solvent for non-mobile elements does not extract the same amount of nutrient as does the root, and any attempt to match the value is futile. The root can contact only a part of the soil, while a chemical solvent can contact all but some internal surfaces. Plant analysis generally provides more current plant-based information than soil testing but it is more costly and entails more effort in terms of sampling, sample handling and analysis. Ideally, both tools should be used as they complement each other.

Unlike soil analysis where the amount of available nutrients are of importance, in plant analysis, the total content of nutrients is relevant. The available nutrients taken from the soil or fertilizer source are assimilated by the plants and, thus, they become part of crop composition.

Knowledge of nutrient concentration in growing plants can serve as a tool for correcting any deficiencies where carried out early enough to safeguard yield. The nutrient uptake by a healthy crop, which has attained its growth and yield potential, is taken as the effective requirement for the crop. It can also be used to evaluate the efficacy of a recent application. The information it provides can help to plan nutrient application in subsequent crops on that field or to compute nutrient removals in relation to productivity and nutrient balance sheets.

Although the nutrient contents vary from one species to another, the composition of a particular species is generally considered to be indicative under optimal nutrient supply and growth conditions. General guidelines of optimal nutrient content in crops can be used for understanding the probable deficiency of nutrients (Table 14).

Whole plant analysis is conducted in order to determine the total nutrient uptake (which is usually carried out on the shoot). For plant analysis to be meaningful as a diagnostic tool, the collection of particular plant parts (tissue) at the right stage of growth for analysis is very important. Plant leaves are considered the focus of physiological activities. The concentrations of leaf nutrients appear to reflect changes in mineral nutrition. As an example, Table 15 shows the specific parts, as identified by various researchers, to be sampled from different plant species. Their concentrations are expected to reflect the true nutrient status of a growing plant (deficiency, sufficiency or excess).

The interpretation of plant analysis data is usually based on the total concentrations of nutrients in the dry matter of leaves or other suitable plant parts compared with standard values of "critical nutrient concentrations" ("critical values"). Between the nutrient concentrations of the deficiency range and those of adequate supply, there is the critical nutrient range. The critical level is that level of concentration of a nutrient in the plant that is likely to result in 90 percent of the maximum yields. The main advantage of critical values, once properly established, is their wide applicability for the same crop. Their disadvantage is that they only provide "yes or no" type of information and do not cover the entire range over which nutrient supplies need to be managed. Table 16 presents some critical values for a range of crops.

TABLE 14
General sufficiency or optimal range of nutrients in plants

Nutrients	Sufficiency or optimal range
Macronutrients	(%)
N	2.0–5.0
P	0.2–0.5
K	1.0–5.0
Ca	0.1–1.0
Mg	0.1–0.4
S	0.1–1.3
Micronutrients	(µg/g)
Zn	20–100
Fe	50–250
Mn	20–300
Cu	5–20
Mo	0.1–0.5
B	10–100

TABLE 15
Typical plant parts suggested for analysis

Crop	Part to be sampled, with age or growth stage
Wheat	Flag-leaf, before head emergence
Rice	3rd leaf from apex, at tillering
Maize	Ear-leaf before tasselling
Barley	Flag-leaf at head emergence
Pulses	Recently matured leaf at bloom initiation
Groundnut	Recently matured leaflets at maximum tillering
Soybean	3rd leaf from top, 2 months after planting
Cotton	Petiole, 4th leaf from apex, at initiation of flowering
Sugar cane	3rd leaf from top, 3–5 months after planting
Tea	3rd leaf from tip of young shoots
Potato	Most recent, fully developed leaf (half-grown)
Tomato	Leaves adjacent to inflorescence (mid-bloom)
Onion	Top non-white portion (1/3 to 1/2 grown)
Beans	Uppermost, fully developed leaves
Pea	Leaflets from most recent, fully developed leaves, at first bloom
Apple, pear	Leaves from middle of terminal shoot growth, 8–12 weeks after full bloom, 2–4 weeks after formation of terminal buds in bearing trees
Cherry	Fully expanded leaves, mid-shoot current growth in July–August
Peach	Mid-shoot leaves, fruiting or non-fruiting spurs, mid-summer leaves
Strawberry	Fully expanded matured leaf without petiole, at peak or harvest period
Banana	Petiole of 3rd open leaf from apex, 4 months after planting
Papaya	3–5-month old leaves from new flush
Pineapple	Middle third portion of white basal portion of 4th leaf from apex, at 4–6-month stage

TABLE 16
Critical nutrient concentrations for 90-percent yield for various crops

Element	Wheat & rice	Oilseed rape	Sugar cane	Alfalfa	Grass*	Citrus
N (%)	3.0	3.5	1.5	3.5	3.0	2.5
P (%)	0.25	0.3	0.2	0.25	0.4	0.15
K (%)	2.5	2.5	1.5	2.0	2.5	1.0
Mg (%)	0.15	0.2	0.12	0.25	0.2	0.2
S (%)	0.15	0.5	0.15	0.3	0.2	0.15
Mn (µg/g)	30	30	20	30	60	25
Zn (µg/g)	20	20	15	15	50	20
Cu (µg/g)	5	5	3	5	8	5
B (µg/g)	6	25	1.5	25	6	25
Mo (µg/g)	0.3	0.3	0.1	0.2	0.3	0.2

Notes:
Growth stage and plant parts used: Wheat/rice: 1–2 nodes, whole shoots; oilseed rape: pre-flowering, youngest mature leaf; sugar cane: 5–7 months, third leaf blade from top; alfalfa: pre-flowering, whole shoots; grass: pre-flowering, whole shoots; citrus: 5–7 month-old leaves from middle of non-fruiting branch.
* Not critical but optimal concentrations for cows producing 15 litres of milk per day.

Critical values determine whether immediate action such as foliar spraying is needed to correct a deficiency. Conclusions can also be drawn as to whether the amount of fertilizer applied at sowing time was sufficient or should be increased for the next crop. Where the concentration is in the toxicity range, special countermeasures are required but no application is called for. For some nutrients, e.g. Ca and Fe, the "active" (mobile) nutrient content of plants should be considered because immobilization can make the total concentrations misleading. The nutrient concentrations of green (fresh) material or of plant sap can be used as a suitable basis for interpretation in some situations.

SAMPLE COLLECTION AND PREPARATION FOR ANALYSIS

Representative sampling should be done of specific plant parts at the growth stage that is most closely associated with critical values as provided by research data. Sampling criteria and procedures for individual samples are similar to those of soil testing in that the sample should be representative of the field. A predetermined, representative number of plants from a homogenous sampling unit contribute to the composition of bulk sample. The composite sample should be about 200–500 g fresh weight. Factors such as the desired precision of recommendation, the nature of the crop (seasonal or perennial) and economic considerations should be taken into account. The following procedure is suggested:

1. For analysis of seasonal crop plants, pick a few representative plants at random from each plot. Remove the shoot (aerial part) with the help of a sharp stainless steel cutter for whole shoot analysis or the desired part for analysis of specific plant parts.
2. If roots are to be included, uproot the whole plant carefully from wet soil, retaining even the fine/active roots. Dip the plant roots gently in water several times to remove adhering soil.
3. Wash with water several times.
4. Wash the samples with about 0.2 percent detergent solution to remove the waxy/greasy coating on the leaf surface.
5. Wash with 0.1M HCl followed by thorough washing with plenty of water. Give a final wash with distilled water.
6. Wash with DDW if micronutrient analysis is to be carried out.
7. Soak to dry with tissue paper.
8. Air-dry the samples on a perfectly clean surface at room temperature for at least 2–3 days in a dust-free atmosphere.
9. Put the samples in an oven, and dry at 70 °C for 48 hours.
10. Grind the samples in an electric stainless steel mill using a 0.5-mm sieve. Clean the cup and blades of the grinding mill before each sample.
11. Put the samples back in the oven, and dry again for constant weight. Store in well-stoppered plastic or glass bottles or in paper bags for analysis.

ANALYTICAL METHODS

The plant sample can be brought into solution form through digestion with acids that dissolve the solid plant parts and bring the plant nutrient in liquid form for estimation. This is called wet digestion. The plant sample can also be heated at high temperatures to destroy OM, and the ash so obtained can be dissolved in acids to bring the sample into liquid form for estimation. This method is called dry ashing.

Wet digestion

A mixture of HNO_3, H_2SO_4 and $HClO_4$ in the ratio of 9:4:1 is used for sample digestion. It is known as tri-acid digestion. When only two acids, viz. HNO_3 and $HClO_4$ (9:4), are used, it is known as di-acid digestion.

Perchloric acid ($HClO_4$) is used primarily for increasing the efficiency of oxidation of the sample as $HClO_4$ dissociates into nascent chlorine and oxygen at high temperature, which increases the rate of oxidation or the digestion of the sample. At times, perchloric acid causes an explosion when it comes into direct contact with the plant sample. Therefore, pre-digestion of the sample with HNO_3 is considered desirable, followed by treatment with the di-acid or tri-acid mixture.

Generally, 1 g of ground plant sample is taken for analysis. It is placed in a 100-ml volumetric flask, and 10 ml of acid mixture is added and the contents are mixed by swirling. The flask is placed on a hotplate in the fumehood and heated, starting at 80–90 °C and then the temperature is raised to about 150–200 °C. Heating continues until the production of red NO_2 fumes ceases. The contents are further heated until the volume is reduced to 3–4 ml and becomes colourless, but it should not be dried. After cooling the contents, the volume is made up with the distilled water and filtered through No. 1 filter paper. This solution is used for nutrient estimation.

Di-acid digestion is used for determination of most of the elements (P, K, Ca, Mg, S, Fe, Mn, Zn and Cu). However, tri-acid digestion is preferred for P and K estimations. It cannot be used for S estimation owing to the presence of H_2SO_4. Sulphuric acid also contains many trace elements as contaminants. Therefore, micronutrients should preferably be estimated through di-acid digestion or by using a dry-ash sample solution.

Wet digestion can also be accomplished by using H_2O_2 to destroy OM followed by digestion with H_2SO_4 to dissolve the sample. In such digestion, N estimation can be carried out as per the Kjeldahl method of total N estimation as described in Chapter 3.

Dry ashing

High-temperature oxidation destroys the OM. The plant sample is ashed at 500–600 °C by placing a suitable weight (0.5–1.0 g) of the sample in a silica crucible and heating it in a muffle furnace for 4–6 hours. The ash residue is dissolved in dilute HNO_3 or

HCl, filtered through acid-washed filter paper in a 50/100-ml volumetric flask, and the volume is made up to the mark. The estimation of K, Ca, Mg and micronutrients (including B and Mo) is carried out in the dry-ashed sample solution.

Dry ashing is a preferred method for the analysis of P, K, Ca, Mg and trace elements, especially B and Mo. It is a relatively simple method and requires very little operational attention. It does not involve the use of perchloric acid. It also avoids the use of boiling acids. However, at times, incomplete recovery of some elements may be caused by:

- Volatilization of elements such as S (also Se and halogens). To avoid loss of S, $Mg(NO_3)_2$ should be mixed with plant samples while dry ashing.
- Retention of elements such as Cu on the walls of silica crucibles. Hence, platinum crucible should be used.
- Formation of compounds that are not completely soluble in the acid used for digestion.

A blank should always be carried out to account for any contamination through the acids used in the digestion.

Nitrogen

Total N in plants is estimated by the Kjeldahl method (Chapter 3). In plants, N is present in protein form, and digestion of the sample with H_2SO_4 containing digestion mixture (10 parts potassium sulphate and 1 part copper sulphate) is required for estimation. Sample size may be 0.5–1.0 g depending on the type of crop and the plant part.

The procedure for sample digestion, distillation and estimation of N is the same as for total N estimation in soil.

Phosphorus

Estimation of total P can be carried out by any of the following methods:

- gravimetric quinolinium phosphomolybdate;
- gravimetric ammonium phosphomolybdate;
- volumetric quinolinium phosphomolybdate;
- volumetric ammonium phosphomolybdate;
- spectrophotometric vanadium phosphomolybdate.

The selection of a method depends on a number of factors, the important ones are:

- speed;
- accuracy;
- reproducibility of results;
- cost of chemicals;
- applicability in the presence of most commonly occurring/interfering cations and anions.

Some of these factors are affected by the quantity and the form of the element to be estimated. Generally, gravimetric methods (which are quite accurate) can be used when the quantity of the element in the sample is quite large.

Therefore, the gravimetric quinolinium phosphomolybdate method is widely used for P estimation in fertilizer samples. The spectrophotometric vanadium phosphomolybdate method is used for P estimation in plant samples where the content is small. Similarly, volumetric methods are also considered suitable for P estimation in plant samples.

Spectrophotometric vanadium phosphomolybdate method

The P content of the plant sample is converted to orthophosphates by digestion with an acid mixture (di-acid or tri-acid). The digested sample is used for P estimation. When orthophosphates are made to react with molybdate and vanadate, a yellow-coloured vanadomolybdophosphoric heteropoly complex is formed. The intensity of the yellow colour is directly proportional to the concentration of P present in the sample, which can be read on the spectrophotometer.

The apparatus required consists of:

- a digestion block;
- a spectrophotometer;
- some beakers/flasks.

The reagents required are:

- Ammonium molybdate – ammonium vanadate in HNO_3 (vanadomolybdate): Dissolve 22.5 g of $(NH_4)_6MO_7O_2.4H_2O$ in 400 ml of distilled water. Dissolve 1.25 g of ammonium vanadate in 300 ml of boiling distilled water. Add the vanadate solution to the molybdate solution and cool to room temperature. Add 250 ml of concentrated HNO_3 and dilute to 1 litre.
- Standard phosphate solution: Dissolve 0.2195 g of analytical-grade KH_2PO_4 and dilute to 1 litre. This solution contains 50 µg P/ml.

The procedure is:

1. Preparation of the standard curve: Put 0, 1, 2, 3, 4, 5 and 10 ml of standard solution (50 µg P/ml) in 50-ml volumetric flasks. Add 10 ml of vanadomolybdate reagent to each flask and make up the volume. The P contents in these flasks are 0, 1, 2, 3, 4, 5 and 10 µg P/ml, respectively. The standard curve is prepared by measuring these concentrations on a spectrophotometer (420 nm) and recording the corresponding absorbances.
2. Take 1 g of plant sample and digest as per the wet digestion method, and make the volume up to 100 ml.
3. Put 5 ml of digest in a 50-ml volumetric flask, and add 10 ml of vanadomolybdate reagent.
4. Make up the volume with distilled water, and shake thoroughly. Keep for 30 minutes.
5. A yellow colour develops, which is stable for days and is read at 420 nm on spectrophotometer.
6. For the observed absorbance, determine the P content from the standard curve.

The relevant calculation is:

$$\text{P content } (\mu g) \text{ in 1 g of sample} = C \times df$$

$$\text{P content (g) in 100 g sample (\% P)} = \frac{C \times df \times 100}{1\,000\,000} = \frac{C \times 1\,000 \times 100}{1\,000\,000} = \frac{C}{10}$$

where:

- ➢ C = concentration of P (µg/ml) as read from the standard curve;
- ➢ df = dilution factor, which is 100 × 10 = 1 000, as calculated below:
  - 1 g of sample made to 100 ml (100 times);
  - 5 ml of sample solution made to 50 ml (10 times).
- ➢ 1 000 000 = factor for converting µg to g.

Potassium

Potassium estimation can be done on a flame photometer, an AAS or by the volumetric sodium tetraphenyl boron method. In a soil/plant analysis laboratory, the use of an AAS is very common and a large number of elements are estimated using this equipment.

Estimation by AAS

The acid-digested or dry-ashed plant sample is used for determining K.

The apparatus required consists of:

- ➢ an AAS;
- ➢ some volumetric flasks.

The reagents required are:

- ➢ Di-acid/tri-acid digestion mixture.
- ➢ KCl (AR-grade) standard solution: Dissolve 1.908 g of pure KCl in 1 litre of distilled water. This solution contains 1 mg K/ml. Take 100 ml of this solution and dilute to 1 litre. This will give 100 µg K/ml as stock solution.
- ➢ KCl working standard solution: Put 5, 10, 15 and 20 ml of stock solution in 100-ml volumetric flasks. Make up the volume. This will give 5, 10, 15 and 20 µg K/ml, respectively.

The procedure is:

1. Set up the AAS and standardize. The relevant parameters for K estimation on an AAS are:
   - ➢ lamp current = 6 m A°;
   - ➢ wavelength = 766.5 nm;
   - ➢ linear range = 0.4–1.5 µg/ml;
   - ➢ slit width = 0.5 nm;
   - ➢ integration time = 2 seconds;
   - ➢ flame = air acetylene.
2. Preparation of the standard curve: Prepare the standard curve using 0, 5, 10, 15 and 20 µg K/ml. The curve will show a linear relationship between the concentration of K and absorbance on a specific wavelength as read from the AAS.

3. Acid-digest 1 g of plant sample and make up to 100 ml. Keep the sample for estimation in the range 5–10 mg K/kg (5–10 µg K/ml) by further diluting as appropriate.
4. Prepare a blank in the same way without adding plant digested material.
5. Take an aliquot of 5 ml for estimation and make up to 100 ml. Atomize on the calibrated AAS, on which the standard curve has also been prepared.
6. Record the absorbance against each sample.
7. From the standard curve, note the concentration of K for the particular absorbance observed for the sample.

The relevant calculation is:

$$\text{K content (µg) in 1 g of sample} = C \times df$$

$$\text{K content (g) in 100 g sample (\% K)} = \frac{C \times df \times 100}{1\,000\,000} = \frac{C \times 2\,000 \times 100}{1\,000\,000} = \frac{C}{5}$$

where:

- C = concentration of K (µg/ml) as read from the standard curve;
- df = dilution factor, which is 100 × 20 = 2 000, as calculated below:
  - 1 g of sample made to 100 ml (100 times);
  - 5 ml of sample solution made to 100 ml (20 times).
- 1 000 000 = factor for converting µg to g.

Sulphur

As dry ashing leads to volatilization loss of S present in the organic combination, and the wet oxidation based on tri-acid mixture includes H_2SO_4, these two methods cannot be used for S determination in plant samples. Therefore, di-acid (HNO_3–$HClO_4$) digestion is used. The turbidimetric method as described for soil S analysis (Chapter 3) is recommended for S analysis of plant samples. The main difference is in the extraction of S. In soils, it is the available S, while in plants, it is the total S, which is brought into solution by di-acid mixture digestion.

The apparatus required consists of:

- a spectrophotometer;
- a mechanical shaker;
- some volumetric flasks.

The reagents required are the same as given for S estimation in soil (Chapter 3), except monocalcium phosphate extracting solution.

The procedure is:

1. Preparation of the standard curve: Same as for S estimation in soil.
2. Digest 1 g of plant sample in di-acid and make the volume up to 100 ml.
3. Transfer 10 ml of the aliquot to a 100-ml volumetric flask.
4. Add 1 g of sieved $BaCl_2$ and shake for 1 minute.
5. Add 1 ml of gum acacia acetic – acid solution, make the volume up to the mark and shake for 1 minute.
6. Run a blank in an identical manner.

7. Measure the turbidity 25–30 minutes after the precipitation at 440 nm.
8. Read the S content in the sample from the standard curve against the similar absorbance as noted for the sample.

The relevant calculation is:

$$\text{S content } (\mu g) \text{ in 1 g of sample} = C \times df$$

$$\text{S content (g) in 100 g sample (\% S)} = \frac{C \times df \times 100}{1\,000\,000} = \frac{C \times \;\; 000 \times 100}{1\,000\,000} = \frac{C}{10}$$

where:

- C = concentration of S (µg/ml) as read from the standard curve;
- df = dilution factor, which is 100 × 10 = 1 000, as calculated below:
  - 1 g of sample made to 100 ml (100 times);
  - 10 ml of sample solution made to 100 ml (10 times).
- 1 000 000 = factor for converting µg to g.

Calcium

Estimation by AAS is described here. However, Ca estimation in the acid digest can also be done by the EDTA titration method (Chapter 3).

The apparatus required consists of:

- an AAS;
- some volumetric flasks;
- a fumehood;
- a hotplate;
- a muffle furnace (when dry ashing has to be done).

The reagent required is:

- Standard Ca solution: Take 0.2247 g of primary standard $CaCO_3$ and add 5 ml of deionized water. Add about 10 ml of HCl to ensure complete dissolution of $CaCO_3$. Dilute to 1 litre with deionized water. This will give Ca solution of 100 µg Ca/ml. Dilute 10 ml of this solution to 100 ml to obtain 10 µg Ca/ml.

The procedure is:

1. Take 1 g of prepared plant sample. Digest in di-acid, and make the volume up to 100 ml.
2. Dilute the sample solution to 10–20 times depending on expected content of Ca, which can be estimated from the standard curve prepared for the purpose.
3. Set up and calibrate the AAS using the relevant parameters:
   - lamp current = 10 m A^0;
   - wavelength = 422.7 nm;
   - linear range = 1–4 µg/ml;
   - slit width = 0.5 nm;
   - integration time = 2 seconds;
   - flame = nitrous oxide acetylene.

4. After setting the AAS, atomize the standard solutions of different concentrations of Ca and record the absorbance for the respective concentrations of Ca. Plot the concentration of Ca on the x-axis and the corresponding absorbance on the y-axis in order to prepare the standard curve.
5. Put 5 ml of the sample solution in a 100-ml volumetric flask and make up the volume, atomize, and observe the absorbance. Note the corresponding concentration for the absorbance recorded that represents the content of Ca in the sample solution.

The relevant calculation is:

$$\text{Ca content (µg) in 1 g of sample} = C \times df$$

$$\text{Ca content (g) in 100 sample (\% Ca)} = \frac{C \times df \times 100}{1\,000\,000} = \frac{C \times 2\,000 \times 100}{000\,000} = \frac{C}{5}$$

where:

- C = concentration of Ca (µg/ml) as read from the standard curve;
- df = dilution factor, which is 100 × 20 = 2 000, as calculated below:
  - 1 g of sample made to 100 ml (100 times);
  - 5 ml of sample solution made to 100 ml (20 times).
- 1 000 000 = factor for converting µg to g.

Magnesium

Estimation by AAS is described here. However, Mg estimation in the acid digest can also be done by the EDTA titration method as described for soils (Chapter 3).

The apparatus required consists of:

- an AAS;
- some volumetric flasks.

The reagent required is:

- Standard Mg solution: Dissolve 10.141 g of $MgSO_4.7H_2O$ in 250 ml of deionized water, and make the volume up to 1 litre. This will give 1 000 µg Mg/ml solution.

Under this procedure, the preparation of the standard curve, the estimation and the calculation procedure are the same as described for Ca estimation (above). The relevant parameters for estimation by AAS are:

- lamp current = 3 m A$^{0;}$
- wavelength = 285.2 nm;
- linear range = 0–0.5 µg/ml;
- slit width = 0.5 nm;
- integration time = 2 seconds;
- flame = air acetylene.

Micronutrients

The estimation of Zn, Mn, Cu, Fe, B and Mo can be done in the same manner as the estimations of K, Ca and Mg (above) using an AAS. However, for B and Mo, chemical methods are preferred, which have been described in Chapter 3. The preparation of the standard curve for each of these metal elements is different and full descriptions are given in Chapter 3. In brief, they are:

- Zn: 1 g of pure zinc dissolved in 20 ml of HCl (1:1) and diluted to 1 000 ml gives 1 mg/ml Zn stock solution. Working solutions can be obtained by diluting the stock solution from 100 to 1 000 times to obtain 10 µg to 1 µg/ml.
- Fe: 1 g of pure iron wire is dissolved in 30 ml of HCl (1:1) and diluted to 1 000 ml to obtain 1 mg/ml of standard Fe. By diluting further, a working solution of a different concentration is obtained.
- Mn and Cu: Standard Mn and Cu solutions are prepared by dissolving 1 g each of pure Mn and Cu metal in 30 ml of HCl (1:1) and making the volume up to 1 000 ml. It will give 1 mg/ml Mn and Cu solutions. Further dilutions can be done to obtain working solutions.
- B: Dissolve 8.819 g of $Na_2B_4O_7.10H_2O$ in warm water. Dilute to 1 litre to obtain 1 mg/ml B stock solution. Dilute further for working solutions and preparation of standard curve.
- Mo: Dissolve 0.15 g of MoO_3 in 100 ml of 0.1M NaOH. Dilute to 1 litre to obtain 1 000 µg/ml or 1 mg/ml of Mo stock solution. Dilute further for working solutions and preparation of standard curve.

Table 17 lists the parameters of different hollow cathode lamps and the fuel to be used for estimation of different elements.

The estimation on an AAS is carried out first by making the standard curve for each element and then estimating the plant sample digested in acid.

The quantity of the element in the sample is calculated by multiplying the concentration observed from the standard curve by the dilution factor. This quantity is present in 1 g of sample taken for estimation. By further multiplying this value by 100, the percentage content is determined.

TABLE 17
Parameters for micronutrient estimation by AAS

Element	Wavelength	Flame	Approximate working range of standard
	(nm)		*(µg/ml)*
Zn	213.8	Air acetylene	0.4–1.5
Fe	248.3	Air acetylene	2.0–9.0
Mn	279.5	Air acetylene	1.0–3.6
Cu	324.7	Air acetylene	1.0–5.0
B	249.7	Nitrous oxide acetylene	1–4
Mo	313.3	Nitrous oxide acetylene	1–4

The relevant example calculation, which is valid for all micronutrients, is:

$$\text{Micronutrient content (µg) in 1 g of sample} = C \times df$$

$$\text{Micronutrient content (g) in 100 g sample (\%)} = \frac{C \times df \times 100}{1\,000\,000} = \frac{C \times 1\,000 \times 100}{000\,000} = \frac{C}{10}$$

where:

- C = concentration of micronutrient (µg/ml) as read from the standard curve;
- df = dilution factor, which is 100 × 10 = 1 000, as calculated below:
  - 1 g of sample made to 100 ml (100 times);
  - 10 ml of sample solution made to 100 ml (10 times).
- 1 000 000 = factor for converting µg to g.

Chapter 5
Water analysis

Irrigation water always contains some soluble salts irrespective of its source. The suitability of waters for a specific purpose depends on the types and amounts of dissolved salts. Some of the dissolved salts or other constituents may be useful for crops. However, the quality or suitability of waters for irrigation purposes is assessed in terms of the presence of undesirable constituents, and only in limited situations is irrigation water assessed as a source of plant nutrients. Some of the dissolved ions, such as NO_3, are useful for crops.

IMPORTANT CHARACTERISTICS OF IRRIGATION WATER

The most important characteristics that determine the quality of irrigation water are:

- pH;
- total concentration of soluble salts assessed through EC;
- relative proportion of Na to other cations such as Ca and Mg, referred to as the sodium adsorption ratio (SAR);
- concentration of B and other elements that may be toxic to plants;
- concentration of carbonates and bicarbonates as related to the concentration of Ca plus Mg, referred to as residual sodium carbonate (RSC);
- content of anions such as chloride, sulphate and nitrate.

The analytical data on the above parameters are used to describe the quality of irrigation water taking standards fixed for each aspect as an index.

Water coming from industries as effluents and domestic wastewater as sewage may contain some specific plant nutrients. These waters may be useful for irrigation of field crops if assessed for toxic/pollutant metals and organic and microbial constituents with regard to their suitability or otherwise. Determination of organic constituents is generally carried out under two categories: (i) organic substances that quantify an aggregate amount of organic C; and (ii) individual or specific organic substances, such as benzene, DDT, methane, phenol and endosulphan. Important determinations are the chemical oxygen demand (COD), which gives the total organic substances, and the biochemical oxygen demand (BOD), which gives the amount of total biodegradable organic substances in the water sample.

The limits of purity established for drinking-water, water for industrial purposes and water for agriculture are different. Therefore, water that is not good for drinking and industrial uses may be suitable for irrigation. However, as the focus of this guide is on service laboratories, it examines only those parameters

of practical utility for assessing the quality of commonly used irrigation waters. Thus, it does not cover aspects pertaining to the use of effluents and sewage waters, nor does it assess water as a carrier of plant nutrients.

Electrical conductivity

The concentration of total salt content in irrigation waters, estimated in terms of EC, is the most important parameter for assessing the suitability of irrigation waters. Generally, all irrigation waters with an EC of less than 2.25 mS/cm are considered suitable except in some unusual situations, e.g. very sensitive crops and highly clayey soils of poor permeability. The ideal value is less than 0.75 mS/cm (Richards, 1954).

Sodium adsorption ratio

The SAR is calculated in order to determine the sodicity or alkalinity hazard of irrigation waters:

$$SAR = \frac{Na^{+}}{\left[\frac{Ca^{2+} + Mg^{2+}}{2}\right]^{1/2}}$$

where the concentration of cations is in me/litre.

Based on the SAR value, waters can be rated into different categories of sodicity (Richards, 1954) as follows:

- safe: < 10;
- moderately safe: 10–18;
- moderately unsafe: 19–26;
- unsafe: > 26.

Residual sodium carbonate

The RSC index is important for carbonate-rich and bicarbonate-rich irrigation waters. It indicates their tendency to precipitate Ca as $CaCO_3$. The RSC is calculated by:

$$RSC\ (me/litre) = (CO_3^{2-} + HCO_3^{-}) - (Ca^{2+} + Mg^{2+})$$

Concentrations of both cations and anions are in me/litre. The sodicity hazard in terms of RSC is categorized (Richards, 1954) as follows:

- safe: < 1.25;
- moderate: 1.25–2.5;
- unsafe: > 2.5.

The limits can vary depending on the type of soil, rainfall and climate conditions. Higher RSC values can be considered safe for sandy soils in a high rainfall area (> 600 mm/year).

Ratio of magnesium to calcium

It is widely reported that Ca and Mg do not behave identically in soil systems, and the Mg deteriorates soil structure particularly where waters are sodium-dominated and highly saline. A high level of Mg usually promotes a higher development of exchangeable Na in irrigated soils. Based on the ratio of Mg to Ca, waters are categorized as:

- safe: < 1.5;
- moderate: 1.5–3.0;
- unsafe: > 3.0.

Boron content

Crops require B in very small quantities. It becomes toxic where present beyond a certain level. In relation to B toxicity, water quality ratings are:

- low hazard: < 1 µg B/ml;
- medium hazard: 1–2 µg B/ml;
- high hazard: 2–4 µg B/ml;
- very high hazard: > 4 µg B/ml.

Each of the above parameters has a bearing on the quality of irrigation water. However, each water source will have its specific suitability or hazardous nature depending on the presence (and the degree) or absence of each of the constituents. Different chemical constituents interact with one another and cause a complex effect on soil properties and plant growth.

Waters with a low SAR and low EC are widely suitable. However, when a value of any one of these parameters (or both) increases in its content, the waters become less suitable for irrigation purposes. The selection of crops for such situations becomes critical. Salt-tolerant crops can be grown in such areas. Soil type is also an important consideration under such situations.

Table 18 indicates the upper permissible limits of EC, SAR, RSC and B for soils with different amounts of clay and for growing tolerant and semi-tolerant crops. These limits are based on extensive trials conducted by Paliwal and Yadav (1976).

TABLE 18
Suitability of irrigation water for semi-tolerant and tolerant crops in different soil types

Texture	Upper permissible limit							
	EC		SAR		RSC		B	
	(dS/m)			*(me/litre)*			*(µg/ml)*	
	ST	T	ST	T	ST	T	ST	T
> 30% clay	1.5	2.0	10	15	2	3	2	3
20–30% clay	4.0	6.0	15	20	3	4	2	3
10–20% clay	6.0	8.0	20	25	4	5	2	3
< 10% clay	8.0	10.0	25	30	5	6	3	4

Note: ST = semi-tolerant crop; T = tolerant crop.

Trace elements

The presence of trace elements or heavy metals reduces crop growth where their concentration increases beyond a certain level in irrigation waters and where such waters are used continuously. However, such elements are not normally a problem in common irrigation waters. They can be of concern where industrial effluent water is used for irrigation.

COLLECTION OF WATER SAMPLES

A representative water sample (500 ml) is collected in a glass or polyethylene bottle, which should be properly washed/rinsed with the same water that is being sampled. Floating debris or any other contaminant should be avoided while collecting the sample. After proper labelling (e.g. source of water, date of collection, and type of analysis required), the sample should be sent to the laboratory without undue delay.

Some of the anions such as SO_4 and NO_3 may be quite low in irrigation waters. Hence, the large volume of the sample has to be first concentrated by evaporating it to about 100 ml in order to obtain their detectable amounts.

ANALYTICAL METHODS

pH

The pH is determined by putting about 50 ml of the water sample in a clean 100-ml beaker and using a pH meter as described in Chapter 3.

Electrical conductivity

A conductivity meter cell is filled with the water sample, and the EC is determined as described in Chapter 3.

Calcium and magnesium

The usual method for determination of Ca and Mg is by versenate (EDTA) titration (Cheng and Bray, 1951). The estimation of Ca and Mg can also be done using an AAS, as described in Chapter 4. Estimation of Ca and Mg in water by the EDTA method is described below.

The apparatus required consists of:

- a porcelain dish;
- some volumetric flasks;
- a burette.

The reagents required are:

- Standard versenate solution (EDTA): Prepare an approximately 0.01N solution of EDTA disodium salt (versenate) by dissolving 2.0 g in distilled water, add 0.05 g of magnesium chloride ($MgCl_2.6H_2O$) and dilute to 1 litre. Standardize against 0.01N calcium chloride solution prepared by weighing 0.500 g AR-grade $CaCO_3$ (oven-dried) and dissolving in minimum excess of dilute HCl (AR), followed by making the volume up to 1 litre with distilled water.

- Ammonium chloride-ammonium hydroxide buffer (pH 10): 67.5 g of pure ammonium chloride dissolved in 570 ml of concentrated ammonium hydroxide and made up to 1 litre; pH adjusted to 10.
- EBT indicator: 0.5 g of EBT and 4.5 g of hydroxylamine hydrochloride (AR) dissolved in 100 ml of 95 percent ethyl alcohol.

The procedure is:

1. Put 5 ml of the water sample in a porcelain dish (8 cm in diameter).
2. Dilute to about 25 ml with distilled water.
3. Add 1 ml of ammonium chloride-hydroxide buffer and 3–4 drops of EBT indicator.
4. Titrate with the standard versenate solution. The colour change is from wine-red to bright blue or bluish green. At the end point, no tinge of the red colour should remain.

From the volume of 0.01N EDTA (standardized against 0.01N $CaCl_2$) solution required for titration, the concentration of Ca and Mg is obtained directly as follows:

$$Ca + Mg\,(\text{me/litre}) = \frac{\text{ml versenate (EDTA) used x normality of EDTA} \times 1000}{\text{ml aliquot taken}}$$

$$Ca + Mg\,(\text{g/litre}) = \frac{Ca + Mg \text{ in me/litre} \times \text{equivalent wt.}}{1000}$$

$$= \frac{Ca + Mg \text{ in me/litre} \times 32.196}{1000}$$

or:

$$Ca + Mg\,(\text{g/litre}) = \frac{\text{ml versenate (EDTA) used} \times \text{normality of EDTA} \times 1\ 000 \text{ x equivalent wt.}}{\text{ml aliquot taken} \times 1000}$$

$$= \frac{\text{ml versenate (EDTA) used} \times \text{normality of EDTA} \times 32\ 196}{\text{ml aliquot taken}}$$

Sodium

A small amount of Na is generally present even in the best-quality irrigation water. The concentration of Na may be quite high in saline water with an EC higher than 1 mS/cm and containing relatively less Ca and Mg. Its estimation is of interest when the water sample tests saline (i.e. an EC higher than 1.0 mS/cm at 25 °C). The determination of Na is carried out directly with the help of a flame photometer using appropriate filters and standard curves prepared by taking a known concentration of Na.

The apparatus required consists of:

- a flamephotometer;
- some volumetric flasks;
- some beakers.

The reagent required is NaCl (AR-grade).

The procedure is:

1. Preparation of the standard curve:

- Take 2.5413 g of NaCl (AR), dissolve in water to make to the volume up to 1 litre; this gives a solution of 1 000 µg Na/ml. From this solution, take 100 ml and dilute to 1 litre to obtain 100 µg Na/ml as stock solution.
- To prepare working standards, take 5, 10, 15 and 20 ml of stock solution in 100-ml volumetric flasks and make up the volume. This gives 5, 10, 15 and 20 µg Na/ml.
- Feed the standards on the flamephotometer one by one in order to obtain a standard curve, with absorbance on the y-axis and the respective concentrations of Na on the x-axis.

2. Feed the water samples on the flamephotometer, and record the absorbance for each sample.
3. Observe the concentration of Na against each absorbance (in µg Na/ml).

The relevant calculation is:

$$\text{Content of Na in mg/litre of water} = \frac{A \times 1\,000}{1\,000} = A$$

where:

- A = absorbance reading (µg/ml) from the standard curve.

It is important to note that:

- If a water sample is diluted for estimation, the quantity of Na as observed on the standard curve is multiplied by the dilution factor.
- If the water sample is concentrated before estimation, the quantity noted from the standard curve is divided by the concentration factor.
- Normally, no dilution or concentrated is required.

Carbonates and bicarbonates

The estimation is based on simple acidimetric titration (Richards, 1954) using different indicators that work in the alkaline (higher than 8.2) or acidic pH range (lower than 6.0).

The apparatus required consists of:

- a porcelain dish;
- a burette.

The reagents required are:

- Phenolphthalein indicator: 0.25 percent solution in 60 percent ethyl alcohol.
- Methyl orange indicator: 0.5 percent solution in 95 percent alcohol.
- Standard sulphuric acid (0.01M).

The procedure is:

1. Put 5 ml of the water sample (containing not more than one milli-equivalent of carbonate plus bicarbonate) in a porcelain dish.
2. Dilute with distilled water to about 25 ml.
3. A pink colour produced with 2–3 drops of phenolphthalein indicates the presence of carbonate, and it is titrated with 0.01M sulphuric acid until the colour just disappears (phenolphthalein end point) because of alkali carbonate having been converted to bicarbonate. This is called the half-neutralization stage. This burette reading (volume used) is designated Y.

4. To the colourless solution from this titration (or to the original sample of water if there was no colour with phenolphthalein), add 1–2 drops of methyl orange indicator, and continue titration with brisk stirring to the methyl orange end point (yellow); the final reading (volume used) is designated Z.

The relevant calculation is:

$$\text{Carbonates (me/litre)} = 2\,(\text{Volume of } H_2SO_4) \times \text{Molarity of } H_2SO_4 \times \frac{1000}{\text{ml of aliquot}}$$

$$= 2Y \times 0.01 \times \frac{1000}{5}$$

$$= 2Y \times 2 = 4Y$$

$$\text{Carbonates (g/litre)} = \frac{2\,(\text{Vol. of } H_2SO_4) \times \text{Molarity} \times 1000 \times \text{Eq.wt.of } CO_3\,(30)}{\text{ml of sample} \times 1000}$$

$$= \frac{2Y \times 0.01 \times 30}{5} = 0\ 12Y$$

The volume of acid used for half-neutralization of carbonate is Y. Hence, for full neutralization it has been assumed as $2Y$:

$$\text{Bicarbonates (me/litre)} = (Z - 2Y) \times \text{molarity of } H_2SO_4 \times \frac{1000}{\text{ml of aliquot}}$$

$$= \frac{(Z - 2Y) \times 0.01 \times 1000}{5}$$

$$= (Z - 2Y) \times 2$$

Where carbonate is absent: $Z \times 2$.

Residual sodium carbonate

This is an important characteristic for assessing the suitability of irrigation water in consideration of a probable sodium hazard. It is calculated from the analysis data for carbonates, bicarbonates and Ca plus Mg as follows (all expressed in me/litre):

$$\text{RSC (me/litre)} = (CO_3^{2-} + HCO_3^-) - (Ca^{2+} + Mg^{2+})$$

Boron

The method for B estimation is same as described in Chapter 3. The determination is carried out using the azomethine-H colorimetric method. It can also be estimated on an AAS. Suitable quantities of the sample should be taken depending on the B content in the waters.

Chloride

Mohr’s titration method is the one most commonly used for chloride estimation. It depends on the formation of a sparingly soluble brick-red silver chromate ($AgCrO_4$) precipitate at the end point when the sample is titrated against standard silver nitrate ($AgNO_3$) solution in the presence of potassium chromate (K_2CrO_4)

as indicator. Initially, the Cl ions are precipitated as AgCl and the dark brick-red precipitate of Ag_2CrO_4 starts just after the precipitation of AgCl has finished.

The apparatus required consists of:

- some beakers;
- a porcelain dish;
- a burette.

The reagents required are:

- Potassium chromate (K_2CrO_4) indicator (5 percent) solution: Dissolve 5 g of K_2CrO_4 in about 75 ml of distilled water, and add saturated solution of $AgNO_3$ drop by drop until a slight permanent red precipitate is formed. Filter and dilute to 100 ml. With high-purity AR, the indicator solution can be prepared directly.
- Standard silver nitrate solution (0.05M): Dissolve 8.494 g of silver nitrate ($AgNO_3$) in distilled water, and make the volume up to one litre. Standardize it against standard NaCl solution, and keep it in an amber-coloured bottle away from the light.

The procedure is:

1. Put 5 ml of the sample in a 100-ml beaker or a porcelain dish, and dilute to about 25 ml with distilled water.
2. Add 5–6 drops of K_2CrO_4 indicator (making it dark yellow), and titrate against the standard $AgNO_3$ solution with continuous stirring until the first brick-red tinge appears.
3. Run a blank to avoid error caused by any impurity in the chemicals.

The relevant calculation is:

$$\text{Cl mg/litre of water} = X \times 1.775 \times \frac{1000}{\text{ml of sample}}$$

where:

- ml of water sample taken = 5;
- X = ml of 0.05M $AgNO_3$ consumed in titration;
- 1.775 = factor representing mg of Cl in aliquot/sample as calculated below:
  - 1 ml of 1M $AgNO_3$ = 1 me of Cl;
  - 1 ml of 0.05M $AgNO_3$ = 0.05 me of Cl = 35.5 × 0.05 = 1.775 mg of Cl (in aliquot).

Sulphate

While traces of sulphate occur universally in all types of waters, its content may be appreciably high in several saline waters showing an EC higher than 1 dS/m at 25 °C. Sulphate can be determined gravimetrically, colorimetrically, turbidimetrically or titrimetrically. Here, the turbidimetric method is described.

Sulphate content is determined by the extent of turbidity created by precipitated colloidal barium sulphate suspension. Barium chloride solid crystals are added to ensure fine and stable suspension of $BaSO_4$ at a pH of about 4.8. This also eliminates interference from phosphate and silicate. This fine suspension of

$BaSO_4$ is stabilized by gum acacia, and the degree of turbidity is measured with a turbidity meter or estimated spectrophotometrically at 440 nm.

The apparatus required consists of:

- a spectrophotometer;
- some beakers;
- some volumetric flasks.

The reagents required are:

- Sodium acetate – acetic acid buffer: Dissolve 100 g of pure sodium acetate in 200 ml of distilled water. Add 31 ml of glacial acetic acid, and make the volume up to 1 litre. Adjust the pH to 4.8.
- Gum acacia: Dissolve 2.5 g of gum acacia in 1 litre of distilled water. Keep overnight and filter.
- Barium chloride: Grind pure $BaCl_2$ crystals to pass through a 0.5-mm sieve but be retained on a 0.25-mm sieve.
- Potassium sulphate solution: To make a stock solution of 10 me S/litre, weigh 1.74 g of pure K_2SO_4 salt and dissolve in 1 litre water.

The procedure is:

1. Put 5 ml of the water sample (of < 1 me S/litre) in a 25-ml volumetric flask. If the EC of water is > 1 dS/m, dilute it with distilled water to bring the EC to lower than 1 dS/m.
2. Add 10 ml of sodium acetate – acetic acid buffer to maintain the pH at about 4.8.
3. Add 1 ml of gum acacia and 1 g of $BaCl_2$ crystals, and shake well.
4. Make the volume up to 25 ml with distilled water.
5. Invert the flask several times, and measure the turbidity with a spectrophotometer at 440 nm using a blue filter.
6. Preparation of the standard curve: For 0, 1, 2, 3, 4 and 5 me S/litre, pipette 2.5, 5, 7.5, 10 and 12.5 ml from stock solution containing 10 me S/litre into 25-ml volumetric flasks. Then develop the turbidity and measure its intensity as in the case of samples. Draw a curve showing S concentration on the x-axis and absorbance on the y-axis.

The S content of the samples is calculated using the standard curve, taking into account the dilution factor of five (5 ml made to 25 ml) and expressed as me S/litre of water.

Nitrate nitrogen

This method depends on the reduction of nitrate to ammonia by adding Devarda's alloy and alkali. The nitrites (NO_2^-) (if present) in the sample are also reduced and determined along with NO_3^--N.

The apparatus required consists of:

- a Kjeldahl distillation assembly;
- an electric muffle furnace;
- a desiccator.

The reagents required are:

- Magnesium oxide (MgO): Heat the MgO at 65 °C for 2 hours in an electric muffle furnace to remove any traces of $MgCO_3$ that may be present. Cool in a desiccator over solid KCl, and store in a tightly stoppered bottle.
- Boric acid with mixed indicator: Weigh 20 g of boric acid, and add about 900 ml of hot distilled water. Cool and add 20 ml of mixed indicator, and make the volume up to 1 litre.
- Mixed indicator: Dissolve 0.066 g of methyl red and 0.099 g of bromocresol green in 100 ml of alcohol.
- Standard sulphuric acid (0.02M).
- Devarda's alloy: Mix Cu, Al and Zn in the ratio of 50:45:5, and grind to pass through a 0.15-mm sieve.

The procedure is:

1. Put 50 ml of the water sample in the distillation flask.
2. Add 0.5 g of MgO and 0.2 g of Devarda's alloy.
3. Put the heaters on and collect the NH_4 (NO_3 converted into NH_4 by reducing agent – Devarda's alloy) in boric acid (20 ml) having mixed indicator into a conical flask, which is connected with the distillation apparatus.
4. Continue distillation to collect about 35–40 ml.
5. Remove the distillate first and then switch off the heating system.
6. Titrate the distillate against 0.02M H_2SO_4 until the pink colour appears.
7. Carry out a blank simultaneously.

The relevant calculation is:

$$NO_3^- - N\ (\text{mg/litre}) = \frac{(X - Y) \times 0.56}{50\ (\text{ml of sample})} \times 1\,000$$

where:

- X = volume (ml) of 0.02M H_2SO_4 consumed in sample titration;
- Y = volume (ml) of 0.02M H_2SO_4 consumed in blank titration;
- 0.56 = factor. 1 litre 1M H_2SO_4 = 28 g N; therefore:

$$1\ \text{ml}\ 0.02\text{M}\ H_2SO_4 = \frac{28 \times 0.02 \times 1000}{1000}\ \text{mg N} = 0.56\ \text{mg N}$$

Chapter 6
Mineral and organic fertilizer analysis

The main objective in analysing fertilizers is to assess their quality. The analysis examines both their physical and chemical composition. The quality of fertilizers is stated by the manufacturers and, in most countries, it is statutorily notified. Hence, analysis is carried out to determine whether the stated quality meets the statutorily notified standards or not. Together with the statutory notified composition of the fertilizers, the testing methods are specified. In situations where the testing methods are not notified, the prevalent standard methods are followed. Generally, the term fertilizer refers to mineral fertilizers, which are manufactured chemical products of standard composition, while the term organic fertilizers refers to organic manures, compost, agro-industrial wastes, etc. The compositions of organic fertilizers, unlike mineral fertilizers, are quite variable and, thus, difficult to regulate precisely.

Fertilizer quality is notified in terms of physical and chemical characteristics. The physical parameters include moisture content and particle size. The chemical parameters refer to the amount and form of nutrients, and to various impurities that may be toxic to plants above a critical limit, e.g. biuret in urea. The efficiency of a fertilizer depends on its form of nutrient content. A phosphatic fertilizer may have water-soluble, citrate-soluble, water-insoluble or citrate-insoluble forms of phosphate. A nitrogenous fertilizer may contain ammoniacal, nitrate and amide forms of N in various proportions.

The accompanying ions also reflect the quality of a fertilizer, such as SO_4 ions in ammonium sulphate, potassium sulphate, superphosphates and many micronutrient fertilizers. They supply S in addition to the main nutrient. Many fertilizers are fortified with micronutrients such as boronated single superphosphate (SSP) and zincated urea.

Therefore, in fertilizer analysis, in addition to estimating total nutrient content, it is necessary to estimate the forms of nutrients and other associated compounds in order to assess their quality properly. For organic fertilizers, the C content and the total content of nutrients are considered relevant and not their forms as they are low-analysis materials.

The analytical methods for fertilizers as described are applicable to most common fertilizers and the forms of nutrient content in them. The procedures as applicable to a particular nutrient could be applicable to any fertilizer with the

nutrient in that particular form. To conform to the scope of this guide, this chapter considers only commonly used solid fertilizers and their major constituents, including impurities.

SAMPLE COLLECTION AND PREPARATION

The collection of a representative sample of a fertilizer is an important step in fertilizer analysis and quality control. The method of sample collection depends on the type and source of fertilizer. Fertilizers are manufactured commodities. They are stored in silos at the manufacturing site for different periods. They are transported using various means (e.g. ship, rail and road) and stored again at various locations including port godowns, warehouses and dealers' shops. Fertilizers are stored/moved both in bulk and as bagged. Therefore, the sample collection methods can be classified broadly as concerning:

- collection from bulk stock in godowns;
- collection from ship hatches or while the ship is being loaded/unloaded;
- collection from bagged stock in godowns of various types;
- sample collection from damaged stock.

Scale of sampling

The number of samples to be collected from a given stock depends on the quantity available in the stock. In a ship, each hold/hatch is treated separately from the others, and samples are collected accordingly. In practice, one representative sample is taken from 100 tonnes of material. A similar scale of sampling is followed for factory silos where bulk fertilizers are stored.

In the case of bagged material, stored in smaller quantities (e.g. at dealers' godowns), generally, 1 sample (minimum) is drawn from 10 bags; 2 samples from 100 bags, 6–7 samples from 1 000 bags; and 10 samples from 2 000 bags. There is no fixed number of samples that can be defined to represent a given quantity. However, it is necessary to ensure that the sample is truly representative of the lot it designates.

In the case of bulk material being loaded into or unloaded from ships, samples are taken from the conveyor belt at certain intervals of time. The material so collected is stored in a container, and from such quantity collected, three representative samples each weighing about 400–500 g are taken.

Samples from the bulk material are collected with the help of sampling cups made of corrosion-free metal. Bagged fertilizers are sampled with the help of a sampling probe/tube. This is often a slotted double tube with a solid cone tip made of stainless steel or brass, about 60–65 cm long, about 1.5 cm in diameter, and with a slot width of about 1.2–1.3 cm. The sampling probe should be inserted diagonally from one corner to another, keeping the slit down and rotated while withdrawing.

The samples collected from the bags or bulk representing one lot is composited and, through the quartering method of leaving aside the portions of two opposite

quarters successively, three identical samples of about 450–500 g each are retained for analysis. These samples are labelled with details showing:

- the name of the fertilizer;
- the source;
- the date when collected;
- the signature, name and details of authority of the person who collected the sample.

Unlike soil, plant and water samples, fertilizer samples are generally collected in triplicate. Of the three samples, one goes to the designated laboratory for analysis; another goes to the owner of the fertilizer (e.g. ship's captain, godown manager, or dealer), and the third sample is kept for further reference by the sample collector. Such a procedure is necessary because in the event of deficiencies in the quality parameters, there has to be provision to analyse the reference sample before holding the party concerned responsible for the poor quality of the fertilizer manufactured/imported/sold to the farmers.

Sample preparation for analysis

The sample received for analysis is recorded in the laboratory with adequate details, and a laboratory code number is assigned in order to identify the sample and to keep its identity confidential.

About half of the sample is ground, sieved through a 1 mm sieve, and stored in a sample bottle for analysis. The remaining half is kept unground for particle size estimation. The samples are stored in an airtight glass bottle or taken for analysis in a moisture-free room (fitted with a dehumidifier) as most fertilizers are hygroscopic in nature.

ANALYTICAL METHODS

There are a number of estimation methods available for each of the constituents. Fertilizer analysis is carried out primarily for quality control and statutory purposes. Each country has adopted certain methods in its fertilizer statute, and only these methods are relevant for that country.

Fertilizer is a widely traded commodity, and it is necessary that the methodology adopted in checking quality be acceptable to all concerned. While there are no internationally accepted or common methods, the methods adopted, verified and notified by the AOAC are widely used (Motsara, 1984). The AOAC adopts a method after its verification and validation by a large number of analytical laboratories in various countries. In view of this, this guide describes the AOAC-based methods of fertilizer analysis in most cases, with simplification of the procedures for ease of comprehension.

Moisture

Two important forms of water present in fertilizers are: (i) absorbed/adsorbed water; and (ii) free water. They are interchangeable depending on the degree of moisture saturation and temperature. Some fertilizers also contain water as an

integral part of their composition, which is referred to as water of crystallization, as in the case of $ZnSO_4.7H_2O$ and $CuSO_4.5H_2O$. As fertilizers are generally hygroscopic in nature, they tend to absorb moisture from the atmosphere (depending on the relative humidity and their packing and storage conditions).

Excessive moisture may damage the granular structure of fertilizers, affect their quality and influence their nutrient content by increasing the weight of fertilizers in a given container.

Therefore, moisture estimation is critical to determining the quality of a fertilizer. The method used depends on the type of fertilizer and the nature of moisture held by it. Some common methods are:

- gravimetric method;
- vacuum desiccator method;
- Karl Fischer titration method.

With the gravimetric or oven-drying method, the loss of water on heating fertilizer samples at a certain temperature is estimated. This method is suitable for fertilizers such as ammonium sulphate, sodium nitrate, superphosphates, muriate of potash (MOP) and sulphate of potash (SOP). It is not suitable for fertilizers that yield volatile substances (such as NH_4) other than moisture on drying at a specified temperature, e.g. calcium ammonium nitrate and di-ammonium phosphate (DAP).

With the vacuum desiccator method, the free moisture present in the fertilizer is absorbed by the desiccant (sulphuric acid), and the loss in weight is reported as moisture. This method is suitable for fertilizers such as calcium ammonium nitrate, DAP, and NPK complexes.

The Karl Fischer titration method is suitable for fertilizers such as nitrophosphates, urea, and urea-based fertilizers, which do not withstand high temperatures.

Gravimetric method

Moisture is estimated by the gravimetric method where the loss in weight at a constant temperature of 100 °C ± 1 °C for 5 hours is measured, e.g. zinc sulphate, and copper sulphate. In the case of sodium nitrate, superphosphates, ammonium sulphate, SOP and MOP, the heating is at 130 °C ± 1 °C. For urea and urea-based fertilizers, the heating is at 70 °C. However, heating at 70 °C does not reflect full moisture content. Therefore, another method such as the Karl Fischer method is preferred.

The apparatus required consists of:

- a glass weighing bottle;
- an electronic balance;
- a temperature-controlled oven.

The procedure is:

1. Weigh 2.0 g of fertilizer sample in a pre-weighed glass weighing bottle.
2. Heat in a temperature-controlled oven for about 5 hours at the specified temperature, as given above for different types of fertilizers.

3. Cool in a desiccator, and weigh.

The relevant calculation is:

$$\text{Moisture \% by weight} = \frac{(B - C) \times 100}{B - A}$$

where:

- A = weight in grams of the empty sample bottle;
- B = weight in grams of the bottle plus material before drying;
- C = weight in grams of the bottle plus material after drying.

Vacuum desiccator method

In this method, the sample is kept in a vacuum desiccator over sulphuric acid. Free moisture present in fertilizers is absorbed by the acid, and the loss in weight of the sample is recorded as the moisture content in the sample.

The apparatus required consists of:

- a vacuum desiccator;
- a porcelain dish;
- a balance.

The procedure is:

1. Weigh (accurately) 5 g of sample in a porcelain dish, and keep it in a desiccator for 24 hours.
2. Take the weight again after 24 hours. The loss in weight is equal to moisture content in the sample.

The relevant calculation is:

$$\text{\% moisture} = \frac{B - C}{B - A} \times 100$$

where:

- A = weight in grams of the porcelain dish;
- B = weight in grams of the porcelain dish plus the fertilizer sample;
- C = weight in grams of the porcelain dish plus the fertilizer sample after desiccation for 24 hours.

Karl Fischer method

The apparatus required consists of:

- a Karl Fischer titrator;
- a balance;
- a beaker or flask;
- a graduated cylinder.

The reagents required are:

- Karl Fischer reagent (pyridine-free).
- Disodium tartrate dihydrate ($Na_2C_4O_62H_2O$) – AR-grade.
- Methanol – Karl Fischer grade / spectroscopy grade containing less than 0.05 percent water.

The procedure is:

1. Standardization of Karl Fischer reagent:
   - Set up the instrument.
   - Add about 25 ml of methanol to the titration vessel until the electrodes are dipped, and titrate with Karl Fischer reagent to a pre-set end point that persists for 30 seconds.
   - Add 100 mg of the disodium tartrate dihydrate to the titration vessel carefully, and titrate with Karl Fischer reagent to a pre-set end point (the end point should persist for 30 seconds). Note the volume (ml) of Karl Fischer reagent used as V_1.
2. Weigh accurately about 1 g of the prepared sample, transfer it carefully to the titration vessel, and stir until dispersed.
3. Titrate with Karl Fischer reagent to the same pre-set end point as above, and note the volume (ml) of Karl Fischer reagent used as V_2.

The relevant calculation is:

$$\text{Moisture \% in sample} = \frac{F \times V_2}{\text{Wt. of sample (g)}} \times \frac{100 \text{ (for \%)}}{000 \text{ (for mg to g)}}$$

where:

- F (factor) in mg H_2O/ml of Karl Fischer reagent =

$$\frac{0.1566 \times \text{mg of sodium tartarate dihydrate added}}{V_1}$$

Disodium tartrate dihydrate contains 0.1566 percent moisture.

Particle size

Fertilizers are manufactured with varying degrees of particle size. This property of fertilizer has a bearing on its efficiency when used in various types of soil for crop production. The size and strength of the particle determine its dissolution time when applied in soil. Most fertilizers are highly water soluble; hence, they dissolve quickly when they come into contact with soil moisture. Fertilizers can be crystalline or granular. With a view to reducing losses caused by rapid dissolution, fertilizers with large granules are also being manufactured, e.g. granular urea and super granular urea.

Granular fertilizers are considered superior for machine application, for preparing bulk blends with greater homogeneity and uniformity, and they are also less vulnerable to adulteration.

Therefore, particle size estimation is an important aspect in determining the fertilizer quality. Most granular fertilizers range between 1 and 4 mm, with a specific particle size for a specific fertilizer.

The apparatus required for particle size estimation consists of sieves of various size.

The procedure consists of sieving through a given sieve size. The material is passed through a sieve with a mesh equal to the maximum particle size prescribed

for a given fertilizer. The material so sieved is retained on a sieve with a mesh equal to the minimum particle size prescribed for that fertilizer. For example, a fertilizer is sieved through a 4 mm sieve and is retained on a 1 mm sieve, kept below the 4 mm sieve. The material retained on the 4 mm sieve is larger than 4 mm in size and that passed through the 1 mm sieve is less than 1.0 mm in size. The material retained on the 1 mm sieve is that with a particle size of between 1 and 4 mm.

Generally, 250 g of the fertilizer is taken and sieved as per the requirement. Sieving can be done mechanically or manually.

Nitrogen

Nitrogen in fertilizers may be present in various forms such as NH_4-N, NO_3-N, urea-N (amide) and organic N. The estimations are carried out for total N and its forms. For urea fertilizer, the total N estimation method is followed. The principle of N estimation is based on the Kjeldahl method. For including or excluding a particular form of N in total N estimation, specific chemicals/catalysts are used. For example, in nitrate-containing fertilizers, 2 g of salicylic acid and 5 g of sodium thiosulphate are added in the digestion mixture. This helps to bind the NO_3-N in the form of nitrosalicylic acid, and it is converted eventually into NH_4-N in the presence of H_2SO_4 and is estimated along with other forms of N present in the sample. Devarda's alloy (2/3 g per sample) can also be used instead of salicylic acid and thiosulphate.

Total nitrogen by the Kjeldahl method

The method and procedure are the same as described for total N estimation in soil (Chapter 3). Specific care is required for sample size. It may vary between 0.2 and 0.5 g depending on the N content of the sample. A smaller amount of sample may be taken for high-analysis fertilizers (e.g. urea) and a larger amount for low-analysis fertilizer (e.g. ammonium sulphate).

Ammoniacal nitrogen by the distillation method

The apparatus required consists of:

- a Kjeldahl distillation unit;
- some flasks, beakers and pipettes;
- a burette.

The reagents required are:

- Freshly ignited carbonate-free MgO.
- Standard acid (0.1M HCl).
- Standard alkali (0.1M NaOH).
- NaOH (40 percent) for distillation.
- Methyl red indicator.

The procedure is:

1. Put 0.5 g of the sample in a 600 ml distillation flask with about 250 ml of water.

2. Add 2 g of freshly ignited carbonate-free MgO or 5 ml of NaOH solution (40 percent) by tilting the flask and through the side of the flask so that the contents do not mix at once.
3. Connect the flask to a condenser by a Kjeldahl connecting bulb and connecting tube.
4. Start heating, and distil about 100 ml of liquid into a measured quantity of standard acid (0.1M HCl).
5. Titrate the distillate with standard NaOH (0.1M) to determine the remaining amount of unused acid, using methyl red indicator. The acid used to neutralize ammonia is equivalent to the N content in the sample.
6. Carry out a blank.

The relevant calculation is:

$$\text{Percent } NH_4 - N = \frac{(A - C) - B}{W} \times 0.0014 \times 100$$

where:

- A = ml of standard acid (0.1M HCl) taken to receive ammonia;
- B = ml of standard alkali (0.1M NaOH) used in titration;
- W = weight of the sample taken;
- C = ml of standard alkali used in the blank.
- 1 ml 0.1M HCl = 0.0014 g N

Ammoniacal plus nitrate-nitrogen by the distillation method

Devarda's alloy (50 percent Cu, 45 percent Al, and 5 percent Zn) reduces NO_3 to NH_4 in an alkaline condition. The method is same as for NH_4-N estimation (above), except that 2–3 g of Devarda's alloy is added before distillation in order to take into account the NO_3 by reducing it to ammonia form.

Nitrate-nitrogen

In fertilizers containing both NH_4 and NO_3-N, first ammoniacal nitrogen is estimated followed by NH_4 plus NO_3 estimation. From the combined value of NH_4 and NO_3, the value of ammoniacal N is subtracted to obtain the nitrate-N content.

Urea nitrogen

The urea form of N can be estimated together with total N by digestion with sulphuric acid. For example, total N is estimated for urea fertilizer. However, for some NPK complexes, urea N has to be estimated separately. In such cases, it is done by the urease method.

The apparatus required for the urease method consists of:

- some beakers;
- some flasks;
- a Gooch crucible;
- some filter paper.

The reagents required are:

- Neutral urease solution: Shake 1 g of jack bean meal with 100 ml of water for 5 minutes. Transfer 10 ml of the solution to a 250 ml Erlenmeyer flask, dilute with 50 ml water, and add 4 drops of methyl purple indicator. Titrate with 0.1M HCl to reddish purple, then backtitrate to green colour with 0.1M NaOH. From the difference in volume used, calculate the amount of 0.1M HCl required to neutralize 10 ml of solution. Based on the calculated acid required, add 0.1M HCl to the remaining 90 ml of solution (about 2.5 ml of acid is required per 100 ml of solution), and shake well.
- HCl (0.1M): Dilute 100 ml of concentrated HCl to 1 litre, and titrate with the standard alkali to establish the exact strength of the acid.
- NaOH (0.1M): Dissolve 4 g of NaOH in 900 ml of water in a 1-litre volumetric flask, make the volume up, and standardize with the standard acid.
- Sodium carbonate (10 percent).
- Barium hydroxide (saturated).

The procedure is:

1. Weigh 10 ± 0.01 g of the sample and transfer it to 15 cm No. 12 fluted filter paper.
2. Leach with about 300 ml of water into a 500 ml volumetric flask.
3. Add 75–100 ml of saturated barium hydroxide solution to precipitate phosphates.
4. Let it settle, and test for complete precipitation with a few drops of saturated barium hydroxide solution.
5. Add 20 ml of 10 percent sodium carbonate solution to precipitate excess barium and any soluble Ca salts.
6. Let it settle, and test for complete precipitation (when the addition of a few more drops of sodium carbonate does not show further precipitation).
7. Dilute to volume, mix, and filter through 15 cm No. 12 fluted paper.
8. Transfer 50 ml of aliquot (equivalent to 1 g of sample) to a 200 or 250 ml Erlenmeyer flask, and add 1–2 drops of methyl purple indicator.
9. Acidify solution with 0.1M HCl, and add 2–3 drops in excess (after colour change is noticed).
10. Neutralize (titrate) solution with 0.1M NaOH to the first change in colour of the indicator.
11. Add 20 ml of neutral urease solution, close flask with rubber stopper, and let it stand for 1 hour at 20–25 °C.
12. Cool the flask in ice water slurry, and titrate at once with 0.1M HCl to full purple colour, then add about 5 ml in excess.
13. Record total volume added, backtitrate excess HCl with 0.1M NaOH to neutral end point.

The relevant calculation is:

$$\%\ \text{Urea} - \text{N} = \frac{(\text{ml } 0.1\text{M HCl} - \text{ml } 0.1\text{M NaOH}) \times 0.0014 \times 100}{\text{Wt. of sample (1 g contained in 50 ml aliquot)}}$$

Biuret

Biuret ($C_2O_2N_3H_5$) is a chemical compound formed by the combination of two molecules of urea with a release of a molecule of ammonia when the temperature during the urea manufacturing process exceeds the controlled level. Fertilizer-grade urea contains biuret, which usually varies between 0.3 and 1.5 percent. Biuret is toxic to plants particularly when applied through foliar spray.

The apparatus required for estimating biuret consists of:

- a water-bath shaker;
- a spectrophotometer;
- some beakers and flasks;
- a burette.

The reagents required are:

- Alkaline tartrate solution: Dissolve 40 g NaOH in 50 ml of cold water and 50 g of $NaKC_4H_4O_6.4H_2O$, and dilute to 1 litre. Let it stand for 1 day before use.
- Copper sulphate solution: Dissolve 15 g of $CuSO_4.5H_2O$ in CO_2-free water, and dilute to 1 litre.
- Biuret standard solution (1 mg/ml): Dissolve 100 mg of reagent-grade biuret in CO_2-free water, and dilute to 100 ml.
- Standard H_2SO_4.

The procedure is:

1. Preparation of the standard curve:
   - Transfer a series of aliquots, 2–50 ml of standard biuret solution, to a 100 ml volumetric flask.
   - Adjust the volume to about 50 ml with CO_2-free water. Add one drop of methyl red, and neutralize with 0.1M H_2SO_4 to a pink colour.
   - Add, with swirling, 20 ml of alkaline tartrate solution and then 20 ml of $CuSO_4$ solution.
   - Dilute to volume. Shake for 10 seconds, and place in a water-bath for 15 minutes at 30 °C ± 5 °C.
   - Also prepare a reagent blank.
   - Determine absorbance of each solution against the blank at 555 nm on the spectrophotometer with a 2.4 cm cell, and plot the standard curve.
2. Stir continuously 5 g of the sample in 100 ml of water for 30 minutes.
3. Filter and wash in 250 ml volumetric flask and dilute to volume.
4. Transfer 25 ml of aliquot to 100 ml volumetric flask and proceed as given under preparation of standard curve.

The relevant calculation is:

$$\text{Biuret \%} = \frac{C \times 100}{W} \text{ x } df$$

where:

- C = concentration in mg/ml of biuret as read from the standard curve;
- W = weight of sample;

- *df* = 200 (5 g of fertilizer extracted to 250 ml, and 25 ml taken for further dilution to 100 ml).

Determination of free acid in ammonium sulphate (as H_2SO_4)

The presence of free acid needs to be regulated in fertilizers such as ammonium sulphate. It is generally kept below 0.025 percent. Its estimation is necessary in order to check the quality of fertilizers.

The apparatus required consists of:

- some beakers and flasks;
- a burette.

The reagents required are:

- Standard sodium hydroxide solution (0.02M).
- Methyl red indicator: Dissolve 0.15 g of water-soluble methyl red in 500 ml of water.
- Methyl red – methylene blue mixed indicator solution: Mix equal volumes of 0.2 percent solution of methyl red and 0.1 percent solution of methylene blue in rectified spirit.

The procedure is:

1. Dissolve about 20 g of the prepared fertilizer sample, accurately weighed, in about 50 ml of cold water.
2. Filter and make the volume up to about 200 ml.
3. Titrate with standard sodium hydroxide solution, using 1–2 drops of methyl red as indicator.
4. If a satisfactory end point (change from red to yellow-orange) with methyl red is not obtained, methyl red – methylene blue mixed indicator may be used.

It is important to use a microburette for this titration. The filtering medium should be neutral and should not contain any alkaline material (which would neutralize free acid).

The relevant calculation is:

$$\text{Free acid as } H_2SO_4 \text{ \% by weight} = \frac{4.9 \times A \times M}{W}$$

where:

- A = volume in millilitres of standard NaOH solution;
- M = Molarity of standard NaOH solution;
- W = weight in grams of prepared sample taken for the test;
- 4.9 = factor (1 M 1 000 ml NaOH = 49 g H_2SO_4; thus, 1 M 100 ml NaOH = 4.9 g H_2SO_4).

Phosphorus

Phosphate (P_2O_5) in fertilizers may be present in different forms: (i) water soluble; (ii) neutral ammonium citrate soluble or insoluble; (iii) citric acid soluble or insoluble; and (iv) acid soluble. Phosphate is generally present as bound with Ca as monocalcium phosphate, dicalcium phosphate and tricalcium phosphate. It may be

present with other metal elements, e.g. aluminium phosphate and iron phosphate. In phosphate rock, it is present as apatite of chlorine and fluorine. Being in water-soluble form, monocalcium phosphate is considered available, while dicalcium phosphate becomes available in slightly acidic situations. Tricalcium phosphate is in an unavailable form and can be available only in acidic situations. Similarly, the aluminium and iron phosphates are also in plant-unavailable forms.

Neutral ammonium citrate soluble form is also considered as available, which includes both monocalcium phosphate and dicalcium phosphate.

In view of the variability in availability to plants, the estimation of different forms of phosphate is critical.

For the so-called "available" forms of P, appropriate extractants have been designed to extract P from fertilizers under a set of well-defined sampling conditions: extractant ratio, temperature, time of extraction, shaking period, etc. The form of P as a fraction of the total P is extracted by a particular method. Estimation of the extracted P utilizes various testing methods: (i) gravimetric; (ii) volumetric; and (iii) colorimetric.

The following methods are used for P estimation in fertilizers:

- gravimetric ammonium phosphomolybdate;
- gravimetric quinolinium phosphomolybdate;
- volumetric ammonium phosphomolybdate;
- volumetric quinolinium phosphomolybdate;
- spectrophotometric vanadium phosphomolybdate.

All the methods are used in various laboratories. For total phosphate estimation, the gravimetric quinolinium phosphomolybdate method is generally preferred because of the minimal interference of other ions and its accuracy and simplicity. Another common method providing acceptable accuracy and simplicity is volumetric ammonium phosphomolybdate.

Gravimetric quinolinium phosphomolybdate method

Various forms of P present in fertilizers are first converted into orthophosphate through chemical treatments. On reaction with quimociac reagent, the orthophosphate precipitates as quinolinium phosphomolybdate [$(C_9H_7N)_3 H_3PO_4.12 MoO_3$] in a boiling medium. The precipitate is weighed gravimetrically, which gives the P content of the sample.

In the gravimetric method, Ca, Fe, Mg, alkali metals and citrates do not affect the analysis. The citrate in the reagent complexes the ammonium ions, thus preventing interference from precipitation of ammonium phosphomolybdate by the ammonium salts usually present in mixed fertilizers. The citrates also reduce interference from soluble silica.

The apparatus required consists of:

- a volumetric flask;
- some beakers;
- a Gooch crucible;
- some filter paper;

- an analytical balance.

The reagents required are:

- Concentrated nitric acid.
- Concentrated hydrochloric acid.
- Magnesium nitrate solution (9 percent): Dissolve 90 g of P-free $Mg(NO_3)_2$ in water, and dilute to 1 litre.
- Acetone.
- Citric acid.
- Sodium molybdate dihydrate.
- Quinoline.
- Quimociac reagent: Dissolve 60 g of citric acid in a mixture of 85 ml of HNO_3 and 150 ml of water, and cool. Dissolve 70 g of sodium molybdate dihydrate in 150 ml of water. Gradually add the sodium molybdate solution to the citric acid – nitric acid mixture, with stirring. Dissolve 5 ml of synthetic quinoline in a mixture of 35 ml of HNO_3 and 100 ml of water. Gradually add this solution to the molybdate citric-nitric acid solution, mix, let it stand for 24 hours, and filter. Add 280 ml of acetone, dilute to 1 litre with water, and mix well. Store in a polyethylene bottle.

According to the nature of the fertilizer, the sample solution should be prepared using one of the following methods:

- For materials and fertilizer mixtures with a high OM content: Put 1 g of the sample in an evaporation dish. Add 5 ml of $Mg(NO_3^-)_2$ solution, and evaporate to dryness. Ignite to destroy the OM, and dissolve in 10 ml of HCl.
- For materials with a low OM content: Put 1 g of the sample in a 50 ml beaker. Add 30 ml of HNO_3 and 5 ml of HCl, and boil gently until the OM is destroyed and red-brown fumes cease to appear.
- For basic slag and fertilizers containing iron or aluminium phosphate: Treat 1 g of the sample with 30 ml of HCl and 10 ml of HNO_3, and boil gently until red-brown fumes disappear.

Cool the solution, prepared by any of the above three methods, dilute to 250 ml, mix, and filter through a dry filter, if required (may contain some insoluble material).

The procedure is:

1. Pipette 5–25 ml of aliquot (sample solution) depending on the P content (containing not more than 25 mg P_2O_5 in the aliquot) into a 250 ml beaker, and dilute to 100 ml with distilled water.
2. Add 50 ml of quimociac reagent, cover with a watch glass, place on a hotplate, and boil for 1 minute.
3. Cool the material to room temperature, swirl carefully 3–4 times during cooling.
4. Filter the precipitate with fibreglass filter paper (or Gooch crucible G4) previously dried at 250 °C and weighed. Wash 4–5 times with 25 ml portions of water. Dry the crucible/filter paper and contents for 30 minutes at 250 °C. Cool in a desiccator to a constant weight.

5. Run a reagent blank with each batch. Subtract the weight of the blank from the weight of the sample precipitate.

The relevant calculation is:

$$\text{Total phosphate (as } P_2O_5\text{) \%} = \frac{3.207(S - B)}{100} \times df \times \frac{100}{W} = \frac{3.207(S - B)}{W} \times df$$

where:

- S = weight of sample precipitate in grams;
- B = weight of blank precipitate in grams;
- df = dilution factor for aliquot taken:
  - suppose volume of the aliquot (solution) taken for estimation = 5 ml, and total volume of fertilizer solution prepared = 250 ml;
  - $df = \frac{\text{Total volume of fertilizer solution}}{\text{Aliquot taken}} = \frac{250}{5} = 50$
- For percentage $= \frac{100}{\text{Wt.}}$
- W = weight of sample taken in grams;
- Factor 3.207 = the quinolinium phosphomolybdate precipitate contains 3.207 percent P_2O_5 on weight basis.

In cases where $MoO_3.Na_2MoO_4.2H_2O$ (quinoline) is not of standard quality, the exact volume of quimociac reagent to be added for precipitation should be calculated by running a series of known standards and observing the phosphate recovery in them.

Volumetric ammonium phosphomolybdate method

Phosphorus is precipitated from the acidic solution as ammonium phosphomolybdate $[(NH_4)_3PO_4.12MoO_3]$ by adding ammonium molybdate solution. The precipitate is dissolved in a measured excess of the standard alkali after filtration and washing until free of the acid.

The apparatus required consists of:

- some volumetric flasks / beakers;
- a burette;
- a shaker;
- a water-bath;
- some No. 44 filter paper.

The reagents required are:

- Magnesium nitrate solution (9 percent): Dissolve 90 g of P-free $Mg(NO_3)_2$ in water, and dilute to 1 litre.
- Concentrated nitric acid.
- Concentrated hydrochloric acid.
- Ammonium molybdate solution (3 percent): Dissolve 30 g of ammonium molybdate in hot distilled water, and make the volume up to 1 litre.
- Standard NaOH solution (0.1M): Dissolve 4 g of NaOH in 1 litre of water, and standardize against standard acid.

- Standard H_2SO_4 solution (0.1M): Take 5.6 ml of concentrated H_2SO_4 and make the volume up to 1 litre. Standardize against a primary standard alkali such as Na_2CO_3 (procedure as detailed in Chapter 3).
- Sodium nitrate (2 percent): Dissolve 20 g of AR-grade sodium nitrate in 1 litre of distilled water.
- Phenolphthalein indicator (1 percent): Dissolve 1 g of phenolphthalein in 100 ml of 95.5 percent ethanol.
- Ammonium nitrate (AR-grade).
- Sodium carbonate (AR-grade).

The sample solution should be prepared using one of the methods indicated for the gravimetric quinolinium phosphomolybdate method (above).

The procedure is:

1. Pipette 5–25 ml of aliquot (sample solution) depending on the P content (containing not more than 25 mg P_2O_5 in the aliquot) in a 250 ml beaker, and dilute to 100 ml with distilled water.
2. Add about 5–10 ml of concentrated HNO_3 and about 10 g of ammonium nitrate.
3. Heat this mixture on a water-bath at 55–60 °C for 10 minutes.
4. Add 3 percent ammonium molybdate solution in the beaker drop by drop with the help of a burette. Continue stirring with a glass rod until about 50 ml of molybdate solution is added. Stir for another few minutes until the yellow precipitate appears to become granular.
5. Cover the beaker with glass and allow it to settle for some time. Decant the clear solution through No. 44 filter paper, and wash the precipitate with 2 percent sodium nitrate solution, agitate thoroughly, and allow the precipitate to settle. Transfer the precipitate to the filter paper, and wash with $NaNO_3$ solution until free from acid (by test with a litmus paper).
6. Transfer the precipitate and filter paper to a beaker, and add 10 ml of 0.1M NaOH at a time by pipette until the precipitate becomes soluble.
7. Add 1–2 drops of 1 percent phenolphthalein, and titrate the excess of alkali against 0.1M sulphuric acid.
8. Run a reagent blank with each batch.

The relevant calculation is:

Total phosphate (as P_2O_5) percent = $F \times (V_1M_1 - V_2M_2) \times df$

$$= 0.0031 \times (40 \times 0.1 - 10 \times 0\ 1) \times \frac{250}{5} \times \frac{100}{1} = 46.5$$

where:

- F = factor for P_2O_5 corresponding to 1 ml of 1M alkali (NaOH). The calculation is as follows: 23 g equivalent of NaOH = 31 g P = 71 g P_2O_5 (P × 2.29 = P_2O_5)

$$1 \text{ equivalent of NaOH or 1000 ml of 1 M NaOH} = \frac{71}{23} \text{ g } P_2O_5$$

$$1 \text{ ml of 1M NaOH} = \frac{71}{23} \times \frac{1}{1000} \text{ g } P_2O_5 = 0.0031 \text{ g } P_2O_5$$

- V_1 = volume of 0.1M NaOH required to dissolve the precipitate (e.g. 40 ml);
- V_2 = volume of 0.1M H_2SO_4 used for titration to neutralize excess alkali (e.g. 10 ml);
- M_1 = molarity of the standard alkali (NaOH);
- M_2 = molarity of the standard acid (H_2SO_4);
- *df* = dilution factor for aliquot taken:
  - suppose, volume of the aliquot (solution) taken for estimation = 5 ml; total volume of fertilizer solution prepared = 250 ml.

$$df = \frac{\text{Total volume of fertilizer solution}}{\text{Aliquot taken}} = \frac{250}{5} = 50$$

- For percentage $= \frac{100}{\text{Wt.}}$

Water-soluble phosphate (P_2O_5)

The water-soluble phosphate is obtained from the sample by dissolving it in distilled water or by washing the sample successively with distilled water. As a procedure, put 1 g of the sample on a filter paper fitted on a 12 cm funnel. Wash with small portions of water at a time to collect about 250 ml of filtrate and make up the exact volume.

Pour water into the funnel only when the earlier portion has drained fully. Otherwise, filtration and complete washing may be prolonged (which should be completed in 1 hour). The filtrate so obtained is used for estimation of phosphate by the gravimetric quinolinium phosphomolybdate method or volumetric ammonium phosphomolybdate method as described above.

The residue remaining on the filter paper contains the water-insoluble portion of P in the sample.

Available phosphate (neutral ammonium citrate-soluble P_2O_5)

For estimating available phosphate, an indirect method is followed whereby total, water-soluble and ammonium citrate-insoluble fractions are estimated. By subtracting citrate-insoluble P from total P, estimates are made for the available P.

The apparatus required for estimation of citrate-insoluble P consists of:

- a volumetric flask / beaker;
- a burette;
- a water-bath-cum-shaker;
- a Büchner funnel.

The reagents required are:

- Concentrated HNO_3.
- Concentrated HCl.
- Concentrated H_2SO_4.
- Ammonium hydroxide.

- Ammonium nitrate (5 percent).
- Quimociac reagent (same as described in total P_2O_5 estimation).
- Filter paper.
- Neutral ammonium citrate solution: Dissolve 370 g of pure citric acid in 1 500 ml of distilled water. Add about 345 ml of 28–29 percent ammonium hydroxide so that the acid is neutralized. After neutralization, the solution must attain a pH of 7.0; if it does not, adjust the pH by adding NH_4OH or citric acid solution.

The procedure is:

1. Follow the procedure as described above for the preparation of a sample solution for estimation of water-soluble phosphate. Within 1 hour, transfer the filter paper and residue to a 250 ml conical flask containing 100 ml of ammonium citrate solution previously heated to 65 °C.
2. Close the flask tightly with a smooth rubber stopper, shake vigorously until the filter paper is transformed to pulp, and release pressure by removing stopper occasionally.
3. Agitate continuously the contents of the stoppered flask in a controlled temperature (65 °C ± 0.5 °C) water-bath-cum-shaker for 1 hour.
4. Exactly 1 hour after adding the filter paper and residue, remove the flask from the shaker, and filter immediately by suction as rapidly as possible through No. 5 filter paper or equivalent, using a Büchner or ordinary funnel.
5. Wash with distilled water at 65 °C until the volume of filtrate is about 350 ml, allowing time for thorough draining before adding more water. If the material is such that it will yield a cloudy filtrate, wash with 5 percent NH_4NO_3 solution.
6. Determine the P_2O_5 in the citrate-insoluble residue (remainder on filter paper) after digestion by one of the following methods:
   - Transfer the dry filter paper and contents to a crucible, ignite until all OM is destroyed. Digest with 10–15 ml of HCl until phosphates are dissolved.
   - Transfer the filter paper and residue to a 250 ml Kjeldahl flask, boil for 30–45 minutes with 30 ml of HNO_3 and 10 ml of HCl. Boil very gently until it is colourless and white dense fumes appear in the flask.
7. Dilute the solution to 250 ml, mix well, and filter through dry filter paper if required. Pipette out 25 ml of aliquot containing not more than 25 mg of P_2O_5 into a 500 ml Erlenmeyer flask, and proceed as described for estimation of total P_2O_5 using quimociac reagent (above).

The relevant calculation is:

$$\text{Citrate insoluble } P_2O_5\% = \frac{3.207\ (S - B)}{100} \times df \times \frac{100}{W}$$

where:

- S = weight of sample precipitate in grams;
- B = weight of blank precipitate in grams;
- W = weight of sample in grams;

- df = Dilution factor $= \frac{\text{Volume of fertilizer solution}}{\text{Aliquot taken}}$
- For percentage $= \frac{100}{W}$

Percent available (citrate-soluble) P_2O_5 = % total P_2O_5 - % citrate-insoluble P_2O_5

The procedure for total P_2O_5 estimation is described above.

Method for estimation of free phosphoric acid (as P_2O_5)

In the specifications of superphosphates, a certain content of free acid (a maximum of 4 percent as phosphoric acid) is allowed in order to check the conversion of water-soluble P into non-water-soluble P during long storage. Therefore, its estimation is essential in determining the quality of superphosphates.

The apparatus required consists of:

- a shaker;
- some flasks and beakers;
- a burette.

The reagents required are:

- Acetone.
- Standard sodium hydroxide solution (0.1M).
- Bromocresol green indicator solution: Dissolve 0.1 g of bromocresol green in 100 ml of rectified spirit.

The procedure is:

1. Weigh accurately about 2.5 g of the prepared sample in a 250 ml Erlenmeyer flask.
2. Add 100 ml of neutral acetone. Fix to a wrist-action shaker. Shake for 1 hour.
3. Filter rapidly through No. 1 filter paper into a 250 ml Erlenmeyer flask, wash with acetone 2–3 times, adding 10 ml of acetone each time.
4. Evaporate the acetone as much as possible.
5. Add about 50 ml of water and drops of bromocresol green indicator.
6. Titrate with standard NaOH solution until the colour changes from yellow to blue.

The relevant calculation is:

$$\text{Free phosphoric acid (as } P_2O_5\text{) percent by weight} = \frac{7.1 \times M \times V}{W}$$

where:

- M = molarity of standard NaOH solution;
- V = volume of standard NaOH solution;
- W = weight in grams of sample taken for the test;
- 7.1 = factor (1 ml 1M NaOH = 0.098 g H_3PO_4; thus, 100 ml 1M NaOH = 9.8 g H_3PO_4. The relationship between H_3PO_4 and P_2O_5 is 196:142 = 0.72. Hence, the factor is 9.8 × 0.72 = 7.1).

Potassium

In all potassic fertilizers, K is generally present in water-soluble form. Therefore, it is estimated directly in fertilizer solution either gravimetrically, volumetrically or flame photometrically. In manures and organic fertilizers, wet digestion with acid is required prior to determination of K in order to bring the element into solution form, as described for plant digestion (Chapter 4).

The methods used for K determination in fertilizers and manures are:

- gravimetric perchloric acid method;
- gravimetric chloroplatinate method;
- gravimetric and volumetric cobaltinitrite method;
- gravimetric and volumetric sodium tetraphenyl boron (STPB) method.

The AOAC-based STPB volumetric method is commonly used in laboratories because of its accuracy and simplicity.

STPB method

Potassium from the fertilizer sample is first extracted with water or ammonium oxalate. The K in extracted solution is precipitated with an excess of STPB as potassium tetraphenyl boron. The excess of STPB is backtitrated with benzalkonium chloride (BAC) or quaternary ammonium chloride using Clayton yellow as indicator: $Na[B(C_6H_5)_4] + K \rightarrow K [B(C_6H_5)_4] + Na^+$.

Interference of NH_4^+ takes place during K precipitation. It is avoided by complexing NH_4^+ with formaldehyde under slightly alkaline conditions before precipitation of K. The chlorides and sulphates do not interfere in the titration.

The apparatus required consists of:

- some volumetric flasks and beakers;
- a burette / semi-microburette;
- some filter paper.

The reagents required are:

- Sodium hydroxide solution (20 percent): Dissolve 20 g of NaOH in 100 ml of distilled water.
- Formaldehyde (HCHO) solution (37 percent).
- STPB solution (about 1.2 percent): Dissolve 12 g of STPB in about 800 ml of water. Add 20–25 g of $Al(OH)_3$, stir for 5 minutes, and filter through No. 42 filter paper (or equivalent) into a 1 litre volumetric flask. Rinse the beaker sparingly with water and add to the filtrate. Collect the entire filtrate, add 2 ml of 20 percent NaOH solution, dilute to volume (1 litre) with water, and mix. Let it stand for 48 hours, and then standardize (as described below). Adjust (by using K salt of known composition for prior standardization by trial and error) so that 1 ml of STPB = 1 percent K_2O. Store at room temperature.
- BAC or quaternary ammonium chloride solution (about 0.625 percent): Dilute 50 ml of 12.8 percent BAC to 1 litre with water, mix and standardize (as described below). If a different concentration is used, adjust the volume accordingly (BAC of 0.625 percent strength is required so the dilution can be done according to the concentration available).

- Clayton yellow (0.04 percent) indicator: Dissolve 40 mg of Clayton yellow powder in 100 ml of water.
- Ammonium oxalate solution [$(NH_4)_2$ C_2O_4] (4 percent): Dissolve 40 g of ammonium oxalate in 1 litre of distilled water.

The procedures for standardizing the solutions are:

- BAC solution: Put 1 ml of STPB solution in a 250 ml Erlenmeyer flask; add 20–25 ml of water, 1 ml of 20 percent NaOH, 2.2 ml of HCHO, 1.5 ml of 4 percent ammonium oxalate, and 6–8 drops of Clayton yellow indicator. Titrate to pink end point with BAC solution, using a 10 ml semi-microburette. Adjust by increasing or decreasing the strength of the BAC solution so that 2 ml = 1 ml of STPB solution (keeping 1 ml STPB = 1 percent K_2O).
- STPB solution: Dissolve 2.5 g of KH_2PO_4 in about 150 ml of water in a 250 ml volumetric flask, add 50 ml of 4 percent ammonium oxalate solution, dilute to volume with water, and mix. Transfer 15 ml of aliquot (51.92 mg of K_2O or 43.10 mg of K) to a 100 ml volumetric flask, add 2 ml of 20 percent NaOH, 5 ml of HCHO and 43 ml of STPB solution. Dilute to volume (100 ml) with water, and mix thoroughly. Let it stand for 5–10 minutes, and then pass through dry No. 42 filter paper. Transfer 50 ml of aliquot of filtrate to a 250 ml Erlenmeyer flask, add 6–8 drops of Clayton yellow indicator, and titrate excess STPB with BAC solution to pink end point. Calculate factor (f) by: f = percent K_2O/ml of STPB solution

$$= \frac{34.61}{43 - \text{ml of BAC used for standardization}}$$

where, 34.61 = % K_2O present in standard KH_2PO_4.

The procedure is:

1. K extraction/preparation of sample solution: Dissolve a known weight (2.5 g) of straight K fertilizer (MOP, SOP, potassium magnesium sulphate) in 200 ml of distilled water, and make the volume up to 250 ml for estimation. For NPK complex fertilizers or NPK fertilizer mixtures, dissolve the sample in 125 ml of water, add 50 ml of 4 percent ammonium oxalate solution, and boil for 30 minutes; after cooling, filter through dry No. 12 filter paper, and make the volume up to 250 ml for further estimation.
2. Transfer 15 ml of aliquot of sample solution to a 100 ml volumetric flask and add 2 ml of 20 percent NaOH and 5 ml of HCHO.
3. Add 1 ml of standard STPB solution for each 1 percent of K_2O expected in the sample plus an additional 8 ml in excess in order to ensure complete precipitation.
4. Dilute to volume (100 ml) with water, mix thoroughly, let it stand for 5–10 minutes, and pass it through No. 12 filter paper (or equivalent).
5. Transfer 50 ml of filtrate to a 250 ml Erlenmeyer flask, add 6–8 drops of Clayton yellow indicator, and titrate excess STPB with standard BAC solution to pink end point.

The relevant calculation is:

$$\% K_2O = (\text{ml STPB used - ml BAC used}) \times f$$

where, f = % K_2O/ml of STPB solution. This factor applies to all fertilizers where 2.5 g of sample is diluted to 250 ml, and 15 ml of aliquot is taken for analysis. To express the results as K rather than K_2O, substitute 28.73 for 34.61 in calculating the value of f.

Micronutrients

Chapter 3 has described the methods for estimating micronutrients in soils using an AAS. In the case of soils, the available micronutrient content is first extracted with the help of appropriate extractants such as DTPA. After standardization of the AAS, the micronutrient content in the extract is estimated. For mineral and organic fertilizers, it is the total content of micronutrients that is relevant. Therefore, organic fertilizers are first acid digested as per the di-acid digestion method described in Chapter 4 (wet digestion method) in order to bring them into solution. From this solution, micronutrient estimations are carried out using an AAS as described in Chapter 3. For mineral fertilizers that are in 100 percent water-soluble form, the estimation of micronutrients is carried out using water-dissolved samples. The NPK complex/compound fertilizers and fertilizer mixtures with water-insoluble ingredients need to be acid digested (similar to organic fertilizers) before analysis is carried out.

Standard curves are prepared for the relevant micronutrients in the same manner as described in Chapter 3. The water-soluble or acid-digested fertilizer samples are used in appropriate quantities so that the content is within the range of concentration used in the standard curve. The nutrient content is calculated taking into account the weight of the sample, the volume made and the aliquot used for estimation.

Organic fertilizers

Unlike soil analysis where available nutrients are estimated, in the case of fertilizers (both mineral and organic) the total contents of nutrients are estimated. In the case of organic fertilizers, the C content and the total content of nutrients are considered relevant and not their forms as they are low-analysis materials. The method for C content estimation is the same as described in Chapter 3. The methods for estimation of total N, P and K in organic fertilizers are the same as described above for mineral fertilizers. With organic fertilizers, the sample always needs to be prepared using the wet-digestion method as described in Chapter 4. The sample size should be 1.0 g (to be weighed exactly).

Chapter 7
Biofertilizer assay and production

Soils are considered dynamic living systems that contain a variety of microbes such as bacteria, actinomycetes, fungi and algae. Bacteria are more numerous than the other three groups combined. They have also been more widely studied and exploited for greater agricultural use than the others.

Maintaining a favourable population of useful microflora is important from a fertility standpoint. The most commonly exploited microbes are those that help in fixing atmospheric N for plant uptake or in solubilizing/mobilizing soil nutrients such as unavailable P into plant-available forms, in addition to secreting growth-promoting substances for enhancing crop yield.

As a group, such microbes are called “biofertilizers” or “microbial inoculants”. They can be generally defined as: preparations containing live or latent cells of efficient strains of N-fixing, phosphate-solubilizing or cellulolytic micro-organisms used for application to seed or soil with the objective of increasing the numbers of such micro-organisms and accelerating certain microbial processes to augment the availability of nutrients in a form that plants can assimilate readily.

Table 19 lists the most commonly accepted specifications of important biofertilizers, when isolated and multiplied for agricultural use.

TABLE 19
Specifications of commonly used biofertilizers

Parameters	*Rhizobium*	*Azotobacter*	*Azospirillum*	Phosphate-solubilizing microbes
Base	Carrier-based			
Cell number at the time of preparation	10^7 cells/g of carrier material or 10^7 cells/ml of liquid material			
Contamination	Nil at 10^5 dilution			
pH	6.5–7.5			
Particle size of carrier material (µm)	150–212			
Moisture (%)	30–40			
Type of carrier	Peat/lignite/charcoal			
Gram stain test	Negative	Negative	Negative	Negative for Pseudomonas and positive for *Bacillus*
Other indicators	Nodule test positive	Minimum amount of N fixed not less than 10 mg/g of sucrose utilized	Should produce white pellicle in N-free bromothymol blue at 10^7 dilution	Should show phosphate-solubilizing zone of 10 mm in tricalcium phosphate medium at 10^6 dilution

Biofertilizers should always be stored in a cool place or at room temperature (25–28 °C), and away from direct heat and sunlight for a longer shelf-life. Shelf-life-expired biofertilizers should not be used. Biofertilizers should not come into direct contact with mineral fertilizers and pesticides.

In order to facilitate understanding of the terms used in this chapter, their descriptions (as per: Motsara, Bhattacharyya and Srivastava, 1995; Bhattacharyya and Tandon, 2002; FAO, 2004), are provided in Annex 13.

TYPES OF MICROSCOPES AND THEIR USE IN THE LABORATORY

The use of microscopes is crucial for biofertilizer identification. Depending on the type of micro-organism and the purpose of study, microscopes of different capacities are used. Microscopes are generally equipped with three objectives: a low-power objective (10×); a high-power objective (40×); and an oil immersion objective (100×). The desired objective is rotated into place. The total magnification obtained with the objectives is as follows:

- The 10× objective with a 10× eyepiece gives a total magnification of 100.
- The 40× objective with a 10× eyepiece gives a total magnification of 400.
- The 100× objective with a 10× eyepiece gives a total magnification of 1 000.

The procedure for using a microscope is:

1. Place the microscope in a position facing the source of light being used, and adjust the mirror to reflect good light into the body tube.
2. Place the slide containing the specimen on the stage.
3. Keeping the eye close to the eyepiece, turn the coarse adjustment slowly to raise the body tube until the slide comes into focus.
4. Turn the fine adjustment slowly until the focus is perfect and the object being examined is seen clearly.
5. Focusing with the oil immersion objective should be done very carefully. For this, first use the low-power objective to locate the portion of the specimen to be examined. Care should be taken to locate the portion in the exact centre of the low-power field as the field diameter is much smaller with the oil immersion objective than with either of the other objectives.
6. Raise the body tube and then rotate the nose-piece until the oil immersion objective clicks into position. Place an oil immersion drop on the portion of the slide directly under the objective. Watch the objective from the side and lower it carefully into the oil. Do not allow the objective to touch the slide. Then study/examine the specimen.

Various types of microscopes used in biofertilizer study are:

- Compound microscope: This microscope consists of two sets of lenses, the objective and the eyepiece. The main functions of the objective are to gather the light rays coming from any point of the object, to unite them in a point on the image, and to magnify the image. The eyepiece magnifies the image further. Most microscope manufacturers have adopted 160 mm as the standard tube length.

- Phase-contrast microscope: In this microscope, light rays passing through an object of a high refractive index are retarded in comparison with light rays passing through a surrounding medium with a lower refractive index. The retardation or phase change for a given light ray is a function of the thickness and the refraction index of the material through which it passes. This microscope has become an extremely important tool in a microbiology laboratory because it intensifies the contrast between translucent objects in unstained living specimens.
- Dark-field microscope: This microscope is so designed that the entering centre light rays are blacked out and the peripheral rays are directed against the object from the side. As a result, the object being viewed appears bright against a dark background. Many micro-organisms that are not visible under bright-field or phase-contrast microscopes can be detected because they reflect light in a dark field and, thus, appear larger than they really are.
- Fluorescent microscope: This microscope is similar to the dark-field microscope except that invisible ultraviolet (UV) light is used to illuminate the object. When exposed to UV radiation, certain substances absorb it and release it almost immediately as visible light of a longer wavelength. The emissions are known as fluorescence and the material as fluorescent.
- Electron microscope: This microscope has a magnifying system that uses a beam of electrons focused in a vacuum by a series of magnetic lenses with a very high detection power. Different types of electron microscopes are available, such as the transmission type (where the image is formed by electrons that pass through the specimen) and the scanning type (where the image formation is based on electrons reflected back from the specimen). The electron microscope has enabled microbiologists to study structures that are too small to be detected by optical microscopes.

EXAMINATION OF MICROBES BY STAINING TECHNIQUES

The morphology of bacteria is examined in two ways: (i) by observing the living, unstained organisms; and (ii) by observing dead cells stained with dyes. The stained preparations of micro-organisms are used to obtain information on the shape, anatomy and taxonomic characters of the cells, which cannot be observed easily in unstained material. The staining can be positive or negative in nature. Positive staining is further classified into simple and differential stains. Positive staining refers to the stain combining with the cell components. In negative staining, the stain does not combine with the cell but forms a deposit on the slide around the cell. The unstained cell appears bright against the darker background of the slide. By this procedure, one can observe the cell components and capsule, which are not easily stained by positive methods.

Before staining, the cells are placed on a microscope glass slide. This preparation is known as a microbiological smear. The slide should be free from grease, otherwise it will interfere with the clarity of the stained preparation. Slides are washed with dichromate-sulphuric acid solution or with detergent to make them grease-free. They are then washed with water, dried, and stored in industrial methylated spirit.

Preparation of smear for staining microbes

A smear is prepared on a microscope glass slide as also on cover glass. The procedure is:

1. Remove the slide from alcohol with the help of forceps. Pass through a Bunsen burner to burn off the alcohol. Allow it to cool.
2. Make a circle about 20 mm in diameter on the slide with the help of a glass marker.
3. Shake the culture tube well and then use a wire loop to take out a drop of the culture, place it on the slide and spread it within the marked circle.
4. Fix the smear by passing the slide through the tip of the blue portion of the flame 4–5 times, and then allow it to cool. The smear is now ready for staining.

Staining

Simple staining

This technique requires only one type of dye for the coloration of a bacterial smear. Methylene blue, crystal violet and carbolfuchsin are the basic dyes. However, they differ in the rate and degree to which they stain a cell. Methylene blue reacts with the negatively charged cells at the slowest rate, taking 30–60 seconds to stain a bacterial cell. Crystal violet is more reactive and a powerful dye. It needs only 10 seconds to stain a cell. Carbolfuchsin is similar to crystal violet in effectiveness.

The reagents required are:

- Methylene blue: This is also known as the Levowitz–Weber modification of the Newman–Lampert stain. Add 0.6 g of certified methylated blue chloride slowly to 52 ml of 95 percent ethyl alcohol and 44 ml of tetrachlorethane (technical grade) in a 200-ml flask. Swirl to dissolve. Let it stand for 12–24 hours at 5–7 °C, then add 4 ml of glacial acetic acid. Filter through No. 42 filter paper (or equivalent) and store in a clean, tightly closed container with a plastic wrapping bag. Traces of water may cause problems with this stain.
- Crystal violet: Dissolve 2 g of crystal violet chloride salt in 20 ml of 90 percent ethyl alcohol and mix with 80 ml of 1 percent aqueous ammonium oxalate.

The procedure is:

1. Prepare smears of two broth cultures. It will save time to make both smears on the same slide, and each should be labelled using a glass marker.
2. Flood the smears with dye (methylene blue or crystal violet solution), and leave on the slide for 2 minutes.
3. Wash the slide to free from stain under a gently running tap, and blot dry with a piece of clean blotting paper.
4. Examine the stained preparation under the oil immersion objective of the microscope (1.8 mm), and make a drawing on a sheet.
5. Observe closely for significant differences in cell size and arrangements.

Differential staining

Differential staining is based on the principle that micro-organisms differ from one another chemically and physically, thus, they react differently to a given staining procedure. This helps in differentiating different types of bacteria. The most common differential stain is Gram stain.

With this technique, when bacteria are stained with certain basic dyes, some species can be decolorized easily with organic solvent such as ethanol or acetone. These are called Gram-negative species. *Rhizobium*, *Azotobacter*, *Azospirillum* and *Pseudomonas* are Gram-negative. Others that resist decolorization are called Gram-positive species (e.g. *Bacillus*). The most commonly used dye is crystal violet.

Negative staining

In negative staining (also known as indirect staining), bacterial cells are not stained but made visible against a dark background. This techniques uses acidic dyes such as eosine and nigrosine. Eosine is used as a soluble salt (sodium eosinate, which ionizes into sodium and eosinate) with the colouring power of dye in the negatively charged eosinate ion. It forms a deposit around the cell, making the bacterial cell appear colourless against a dark background. The negative staining method is not so common because it leaves the cell colourless. However, its advantage over direct staining is that it gives a more accurate view of the bacterial cell.

The reagents required are:

- Eosine solution: Dissolve 2 g of sodium eosinate salt in 50 ml of hot water.
- Nigrosine solution: Dissolve 10 g of nigrosine salt in 100 ml of hot water.

The procedure is:

1. Place a loopful of bacterial suspension on a glass slide, and put a drop of 1 percent aqueous solution of the dye. Mix thoroughly with the help of a sterile wire loop.
2. Spread the mixture uniformly on the slide.
3. Allow the slide to air dry. Do not heat it. Examine it under the oil immersion objective, and make drawing on a sheet. The area around the cells will appear dark blue, and the cells themselves will be colourless.

The layering of the staining solution should be thin. Otherwise, it will not allow light to pass through the object, and the dye will crack on drying. However, if the layer (film) is too thin, it will not give a good contrast.

CULTURE MEDIA

In nature, microbes are found as a mixed population. The types that encounter the most favourable environmental and other growth conditions emerge in abundance while others that find the environment less favourable are fewer in number. However, for any kind of study on them or for their use as a pure species, they have to be separated from other species. The nutritional media or culture media are different for different species. The culture media may be of two types: liquid, without the use of agar; and solid, containing agar. The cultures may be: pure

(contain only one species); mixed (contain two or more species); or contaminated (contain unwanted species along with the intended species).

As the medium is specific for certain species, it checks or restricts the growth of other microbes for which it may not be suitable. However, some growth of and contamination by unwanted species may occur. Therefore, isolation of specific microbes is necessary for their greater efficiency in use.

Types of culture media

The media may be either empirical or synthetic.

Empirical media are natural media (usually meat extract) that provide all the nutritive substances such as N, C, proteins, amino acids, and mineral salts. Along with the meat extract, peptone, sodium chloride (for correct osmotic pressure) and phosphate salts (as buffer to stabilize pH) are also added to the medium. The exact chemical composition of the media remains unknown.

Synthetic media are prepared with known types and quantities of the chemical compounds. They contain a source of C and N together with other nutrients such as sodium and potassium phosphates, magnesium sulphate and calcium chloride. Molybdenum is also usually added. Treptophan (an amino acid) is also used as an N source. The absence of a particular nutrient source restricts the growth and multiplication of the micro-organisms.

Preparation of culture media

There are specific media for specific types of microbes. No single medium can help grow all types of microbes.

In preparing the culture medium, the contents specified for the purpose are weighed and mixed in a conical flask. Water is added and then heated to dissolve the contents, and the solution is stirred. The pH value is adjusted to the desired level. Precipitation is avoided during the preparation.

In the case of a solid medium (one containing agar), the container is placed in such a way that the maximum surface area is available for the growth of micro-organisms. For this purpose, slanting tubes, conical flasks and Petri dishes are considered suitable.

The culture medium and the glassware are sterilized in an autoclave in which the steam under pressure is maintained at 1–1.25 kg/cm^2 and the heating is done for 15–30 minutes at a temperature of 121 °C.

For a medium containing heat-sensitive materials (e.g. sugars, amino acids and vitamins), sterilization is done by passing the medium through different types of filters (asbestos or sintered glass) that are capable of retaining bacteria (where present as a contaminant).

Certain chemicals, such as salts of heavy metals ($AgNO_3$, $HgCl_2$, and $KMnO_4$), halogens (chlorine, bromine, iodine and their salts) and organic compounds are also used for sterilization of the medium.

Table 20 lists the most commonly used media for various micro-organisms.

TABLE 20
Micro-organism-specific media

Micro-organism	Media	Author
Bacteria (general)	Thornton's agar medium	Thornton (1922)
Rhizobium	CRYEMA (Congo red yeast extract mannitol agar)	Vincent (1970)
	Norris and Date medium	Norris & Date (1976)
Azotobacter	Jensen's N-free medium	Jensen (1942)
	Ashby's medium	Ashby (1907)
	Beijerinckia's medium	Becking (1959)
Azospirillum	Semi solid malate medium	Baldini & Dobereiner (1980)
	Nitrogen free bromothymol blue medium	Dobereiner, Marriel & Nery (1976)
	Okon's modified medium	Okon, Albrecht & Burris (1977)
PSMs	Pikovskaya medium	Pikovskaya (1948)

The composition and method of preparation of the media listed in Table 20 are described below.

Thornton's agar media

The composition of the medium is:

- mannitol: 1.0 g;
- asparagine: 0.5 g;
- K_2HPO_4: 1.0 g;
- KNO_3: 0.5 g;
- $MgSO_4.7H_2O$: 0.2 g;
- $CaCl_2$: 0.1 g;
- NaCl: 0.1 g;
- $FeCl_3$: 0.002 g;
- agar: 15.0 g;
- distilled water: 1 000 ml.

To prepare the medium, dissolve phosphate, nitrate and asparagine in distilled water, add magnesium sulphate, calcium chloride, sodium chloride and ferric chloride. Add agar and dissolve, heat at 100 °C, and filter. Add mannitol, and cool to 60 °C. Adjust the pH to 7.4 with bromothymol blue, and autoclave at 121 °C for 30 minutes at 1–1.25 kg/cm^2 pressure. Cool, and store for use.

Congo red yeast extract mannitol agar medium

The composition of the medium is:

- K_2HPO_4: 0.5 g;
- $MgSO_4.7H_2O$: 0.2 g;
- NaCl: 0.1 g;
- mannitol: 10 g;
- yeast extract: 1 g;

- agar: 15.0 g;
- distilled water: 1 litre;
- 1 percent aqueous Congo red: 2.5 ml.

To prepare the medium, dissolve the salts in water, add agar, adjust the pH to 7.0, and autoclave (as above).

Glucose peptone agar medium

The composition of the medium is:

- glucose: 5.0 g;
- peptone: 10.0 g;
- agar: 15.0 g;
- distilled water: 1 litre.

To prepare the medium, dissolve the salts in water, add agar, adjust the pH to 7.0, and autoclave (as above).

Norris and Date liquid medium

The composition of the medium is:

- mannitol: 10.0 g;
- yeast extract: 1 g;
- K_2HPO_4: 0.5 g;
- $MgSO_4.7H_2O$: 0.8 g;
- NaCl: 0.2 g;
- $FeCl_3.6H_2O$: 0.01 g;
- distilled water: 1 litre.

To prepare the medium, dissolve the salts in water, adjust the pH to 7.0, and autoclave (as above).

Jensen's N-free medium

The composition of the medium is:

- sucrose: 20.0 g;
- K_2HPO_4: 1.0 g;
- $MgSO_4.7H_2O$: 0.5 g;
- NaCl: 0.5 g;
- $FeSO_4$: 0.1 g;
- $CaCO_3$: 2.0 g;
- agar: 15.0 g;
- distilled water: 1 000 ml.

To prepare the medium, dissolve the salts in water, add agar, adjust the pH to 7.0, and autoclave (as above).

Ashby's medium

The composition of the medium is:

- mannitol: 20.0 g;
- K_2HPO_4: 0.2 g;

- $MgSO_4$: 0.2 g;
- NaCl: 0.2 g;
- K_2SO_4: 0.1 g;
- $CaCO_3$: 5.0 g;
- agar: 15.0 g;
- distilled water: 1 000 ml.

To prepare the medium, dissolve the salts in water, add agar, adjust the pH to 7.0, and autoclave (as above).

***Beijerinckia* medium**

The composition of the medium is:

- sucrose: 20.0 g;
- KH_2PO_4: 0.8 g;
- K_2HPO_4: 0.2 g;
- $M_gSO_4.7H_2O$: 0.5 g;
- $FeCl_3$: 0.1 g;
- Na_2MoO_4: 0.005 g;
- agar: 15 g;
- distilled water: 1 000 ml.

To prepare the medium, dissolve the salts in water, add agar, adjust the pH to 6.5, and autoclave (as above).

Semi-solid malate medium

The composition of the medium is:

- malic acid: 5.0 g;
- K_2HPO_4: 0.5 g;
- $MgSO_4.7H_2O$: 0.2 g;
- NaCl: 0.1 g;
- $CaCl_2$: 0.02 g;
- $Na_2MoO_4.2H_2O$: 0.002 g;
- $MnSO_4.H_2O$: 0.01 g;
- KOH: 4.5 g;
- biotin: 0.1 mg;
- Fe-EDTA (1.64 percent): 4.0 ml;
- bromothymol blue: 3.0 ml (0.5 percent alcoholic solution);
- distilled water: 1 000 ml.

To prepare the medium, dissolve the salts in water, adjust the pH to 6.8, and autoclave (as above).

Nitrogen-free bromothymol blue medium

The composition of the medium is:

- malic acid: 5.0 g;
- KOH: 4.0 g;
- K_2HPO_4: 0.5 g;

- $FeSO_4.7H_2O$: 0.05 g;
- $MnSO_4.H_2O$: 0.01 g;
- $MgSO_4.7H_2O$: 0.01 g;
- NaCl: 0.02 g;
- $CaCl_2$: 0.01 g;
- Na_2MoO_4: 0.002 g;
- distilled water: 1 litre;
- bromothymol blue: 2.0 ml (0.5 percent alcoholic solution);
- agar: 1.75 g.

To prepare the medium, dissolve the salts in water, add agar, adjust the pH to 6.6–7.0, and autoclave (as above).

Okon's modified liquid medium

The composition of the medium is:

- Part (i):
  - K_2HPO_4: 6.0 g;
  - KH_2PO_4: 4.0 g;
  - distilled water: 500 ml.
- Part (ii):
  - $MgSO_4$: 0.2 g;
  - NaCl: 0.1 g;
  - $CaCl_2$: 0.02 g;
  - NH_4Cl: 1.0 g;
  - malic Acid: 5.0 g;
  - NaOH: 3.0 g;
  - yeast extract: 0.05 g;
  - Na_2MoO_4: 0.002 g;
  - $MnSO_4$: 0.001 g;
  - H_3BO_3: 0.0014 g;
  - $Cu(NO_3)_2$: 0.0004 g;
  - $ZnSO_4$: 0.0021 g;
  - $FeCl_3$: 0.002 g;
  - distilled water: 500 ml;
  - bromothymol blue: 2 ml (0.5 percent alcoholic solution).

To prepare the medium, dissolve and sterilize parts (i) and (ii) separately, and mix aseptically while hot.

Pikovskaya medium

The composition of the medium is:

- glucose: 10.0 g;
- $Ca_3(PO_4)_2$: 5.0 g;
- $(NH_4)_2SO_4$: 0.5 g;
- NaCl: 0.2 g;
- KCl: 0.2 g;

- $MgSO_4.7H_2O$: 0.1 g;
- $MnSO_4$: traces;
- $FeSO_4$: traces;
- yeast extract: 0.5 g;
- agar: 15.0 g;
- distilled water: 1 litre.

To prepare the medium, dissolve the salts in water, add agar, and autoclave (as above).

ISOLATION AND IDENTIFICATION OF IMPORTANT MICROBES

Rhizobium

A well-established symbiotic relationship exists between *Rhizobium* species and leguminous plants, as a result of which elemental N is fixed or converted to ammonia. A specific *Rhizobium* culture for a specific legume crop that has a high ability for infection, nodulation, N_2-fixation and antibiotic resistance is needed. *Rhizobium* it is an aerobic and gram-negative microbe, and abundant in the root nodules of leguminous crops.

Isolation

Rhizobium strains can be isolated from the root nodules of legumes as follows.

The apparatus required consists of:

- an incubator;
- sterilized water;
- a Petri dish;
- a glass rod;
- a test-tube (sterilized);
- an inoculation needle.

The reagents required are:

- Ethyl alcohol (95 percent).
- Mercuric chloride (0.1 percent weight/volume).
- Congo red yeast extract mannitol agar (CRYEMA) plate.

The procedure consists of two parts:

- Nodule selection and surface sterilization:
  1. Uproot a healthy legume plant from which a *Rhizobium* strain is required.
  2. Wash the roots of the plant gently under tap-water.
  3. Select pink-coloured healthy nodules, and separate them from the root with the help of a sharp clean blade.
  4. Put the nodules in a test-tube, and wash them thoroughly 4–5 times with tap-water so that the surface of the nodules becomes soil-free.
  5. Immerse undamaged nodules in 95 percent ethyl alcohol for 5–10 seconds, and rinse them with sterilized water.

6. Transfer the nodules to a sterile test-tube, and keep the nodules immersed in 0.1 percent mercuric chloride solution for 2–3 minutes.
7. Shake the test-tube from time to time in order to remove air bubbles from the surface of the nodules and to bring fresh sterilant in contact with the surface of the nodules.
8. Decant off the mercuric chloride solution, and flood the nodules with sterilized water 5–8 times in order to remove all the sterilant.

➢ Plating of nodule suspension:

1. Add a few drops of sterilized water to the test-tube, and crush the nodules in the tube with the help of a sterile glass rod.
2. The suspension so formed has a very high count of bacteria. Dilute this suspension by adding 2–4 ml of sterilized water to the tube.
3. Take one drop or 0.1 ml of the suspension, and put it on a plate containing CRYEMA medium.
4. Spread the suspension over the plate gently with the help of a sterile glass spreader. Repeat this spreading for three different plates containing the same medium.
5. Invert the plates, and keep them for incubation at 28–30 °C until small, elevated, round-shaped colonies develop. If the colonies are white, translucent, glistening and elevated, they may be expected to be colonies of *Rhizobium*. Any colony absorbing the red colour of the Congo red indicator may be assumed to be contaminated.

After proper identification, *Rhizobium* from the colony so developed can be taken and multiplied in order to obtain a pure culture through the inoculation and fermentation process.

Identification

The following culture tests need to be carried out in order to check whether the *Rhizobium* colony as developed in the above incubation is pure or contaminated with common contaminants such as *Agrobacterium*.

➢ Growth in alkaline medium: *Agrobacterium radiobacter* can be detected by drawing streaks on Hoffer's alkaline medium (pH 11.0) where *Rhizobium* does not grow, while *A. radiobacter* does.

➢ Growth in glucose peptone agar: *Rhizobium* shows little or no growth on glucose.

➢ Gram test: It should test negative.

Azotobacter

Azotobacter is a non-symbiotic, aerobic, free-living bacteria. It is capable of fixing N and synthesizing growth-promoting substances and vitamins. It is found mainly near the rhizosphere. In soil, its population varies but rarely exceeds 10^2–10^3/g soil.

Isolation

The apparatus required consists of:

- an incubator;
- some test-tubes;
- some Petri dishes;
- some glass rods;
- some inoculation needles;
- some pipettes;
- some conical flasks.

The reagents required are:

- sterile water;
- Jensen's medium.

The microbes are isolated from the soil sample (collected from the rhizosphere) by the soil dilution method and plating on an N-free agar medium. The procedure is:

1. Collect moist soil from the rhizosphere, and make a suspension of 10 g of this soil in 90 ml of sterile water in a conical flask.
2. Shake the suspension for about 5 minutes.
3. Dilute this suspension serially as follows:
   - Arrange at least 6 test-tubes, each containing 9 ml of sterile water, in a test-tube stand.
   - Take 1 ml of the suspension from the conical flask aseptically and add to the first test-tube containing 9 ml of sterile water. Total volume will be 10 ml. Shake the test-tube vigorously.
   - Pipette out 1 ml of the suspension from this test-tube and add it to the second test-tube and shake.
   - Repeat this process serially until the last test-tube.
4. Pipette out 1 ml of liquid aseptically from the third, fourth, fifth and sixth test-tubes (to start with a minimum dilution of 1 000 times), and pour onto separate N-free agar medium (Jensen's medium) plates. Spread the aliquot on the plates, and mark the plates.
5. Invert the plates, and incubate them at about 28 ± 2 °C for at least three days.
6. After incubation, soft, flat, transparent or milky, mucoid colonies of *Azotobacte*r will develop on the plates.
7. Pick up a single colony aseptically with the help of a sterilized inoculation needle and streak it on an N-free agar media plate.
8. Invert and incubate the plate at 28 ± 2 °C. In this way, pure colonies of *Azotobacter* will be formed.

After proper identification, *Azotobacter* from the colony so developed can be taken and multiplied in order to obtain a pure culture through the inoculation and fermentation process.

Identification

Azotobacter has the ability to produce pigment. Different species of *Azotobacter* produce different pigments, thus facilitating their identification. The pigments produced by important species are:

- *Azotobacter chroococcum* – brown;
- *Azotobacter beijerinckii* – light brown;
- *Azotobacter vinelandi* – greenish yellow;
- *Azotobacter insignis* – light brown;
- *Azotobacter agilis* – green;
- *Azotobacter macrocytogenes* – purple.

Although pigment production does not give a confirmation of the species, it is a good indication of their presence.

The secretion of gum or polysaccharide is another important characteristic, as is the formation of cysts by all the species (for withstanding adverse conditions).

Azospirillum

Azospirillum is a spiral-shaped N-fixing bacteria. It also produces hormones and vitamins. Important species are *Azospirillum brasilense* and *Azospirillum lipoferum*. It is widely distributed in soils and grass roots.

Isolation

As *Azospirillum* bacteria occur inside as well as outside plant roots, plant roots are taken in order to isolate *Azospirillum*.

The apparatus required consists of:

- an incubator;
- sterilized water;
- a Petri dish;
- a glass rod;
- a test-tube (sterilized);
- an inoculation needle.

The reagents required are:

- Ethyl alcohol.
- 0.1 percent mercuric chloride.
- Phosphate buffer.

The procedure is:

1. Take roots of any field crop plants.
2. Wash the roots first under tap-water, and then surface sterilize with 95 percent ethyl alcohol for about 5 seconds.
3. Cut the roots into small pieces (0.5 cm long), and wash them with 0.1 percent mercuric chloride for 1 minute, followed by washing with sterile water and then with phosphate buffer.
4. Place the pieces of roots in screw-capped tubes containing semi-solid sodium or calcium malate medium, and incubate at 28–30 °C for 3–4 days.

5. After proper incubation, a white pellicle of *Azospirillum* will develop 1–2 cm below the upper surface of the medium.
6. Transfer the isolate (white pellicle) 3–4 times to semi-solid calcium malate medium contained in screw-capped tubes.
7. Thereafter, make serial dilutions up to 10^{-10}, and then incubate 0.1 ml from the last dilution to a fresh tube/plate containing semi-solid calcium malate medium for growth.
8. Incubate the tube/plate for 3–4 days. Observe the growth of *Azospirillum*.

After proper identification, *Azospirillum* from the colony so developed can be taken and multiplied in order to obtain a pure culture through the inoculation and fermentation process.

Identification

Azospirillum organisms are Gram-negative, curved, and rod-shaped of varying size. They contain poly-B-hydroxybutyrate granules. *Azospirillum* shows spiral movements. The formation of white pellicles on semi-solid calcium malate medium is a characteristic of *Azospirillum*. *Azospirillum* forms round-shaped colonies on the solid malate medium. *Azospirillum* micro-organisms are producers of strong bases. Hence, when they grow in a medium containing bromothymol blue indicator, they change the colour of the medium to blue.

Phosphate-solubilizing micro-organisms

Soil bacteria of the genera *Pseudomonas*, *Bacillus* and certain fungi of the genera *Penicillium* and *Aspergillus* possess the ability to solubilize insoluble forms of P owing to their secretion of organic acids. These microbes help in the solubilization of P from phosphate rock and other sparingly soluble forms of soil P by reducing their particle to nearly amorphous forms. They are present in abundance in the rhizosphere. The phosphate-solubilizing micro-organisms (PSMs) are isolated directly from the soil by the soil serial dilution method.

Isolation

The apparatus required consists of:

- an incubator;
- sterilized water;
- a Petri dish;
- a glass rod;
- a test-tube (sterilized);
- an inoculation needle.

The reagent required is sterile water.

The procedure is:

1. Take 10 g of soil, and dilute it serially by the same method as described for *Azotobacter* (above).
2. Take 4–5 plates containing Pikovskaya medium, which contains insoluble phosphate.

3. Take 1 ml of liquid soil suspension from 3rd, 4th, 5th and 6th test-tubes and pour aseptically onto the plates/Petri dishes containing the medium. Soil suspensions with different dilutions will ensure the growth of micro-organisms depending on their population and, thus, could be taken for further purification and multiplication from the appropriate tube.
4. Spread the aliquots (soil suspension) of different dilutions on the plates.
5. Invert the plates, and incubate them at 28–30 °C for 3–4 days.
6. Microbial colonies with a transparent zone will develop.
7. Take a single colony and streak it on phosphate-containing medium plates, and incubate at 28 °C for 3–4 days.
8. Take the single colony from the above plates having clear zones of solubilization, and maintain them on slants, as this provides a larger surface area for growth.

After proper identification, PSMs from the colony so developed can be taken and multiplied in order to obtain a pure culture through the inoculation and fermentation process.

Identification

Phosphate-solubilizing micro-organisms can be either bacteria or fungi. The following species are more effective:

- bacteria:
  - *Bacillus megaterium*,
  - *Bacillus polymyxa*,
  - *Bacillus pulvijaciens*,
  - *Pseudomonas striata*,
  - *Pseudomonas rathonis*;
- fungi:
  - *Aspergillus niger*,
  - *Aspergillus awamori*,
  - *Penicillium digitatum*.

The bacterial species are aerobic and heterotrophic. Cell size is 1.1–2.2 µm, and the cells are rod-shaped. The transparent zone around microbial colonies indicates the extent of phosphate solubilization and the effectiveness of the microbes.

INOCULATION OF CULTURE MEDIUM

This is a method by which micro-organisms are transferred from any source (purified culture) to the medium for their multiplication in a laboratory. To avoid any contamination, inoculation is done in a laminar air-flow chamber aseptically as follows:

1. Select a pure culture of the micro-organism to be multiplied.
2. Take a specific culture medium in a sterilized flask/tube (already prepared and kept ready).
3. Hold the pure culture tube between thumb and forefinger in such a way that the cotton plug of the tube is towards the body of the worker.

4. Remove the cotton plug from the tube/flask containing the culture, take the pure culture from the tube using a sterilized platinum needle and transfer it to the medium in the flask/tube. Plug the inoculated tube/flask, keep it in the incubator at 28 ± 2 °C for 3–5 days to allow the microbes to grow. When a large volume of culture medium is required for mass production of biofertilizer, multiplication is done in large flasks (2–5 litres) in a rotary shaker or in fermenters.

FERMENTATION

The sterilized medium is inoculated with a pure culture of the desired micro-organism as described above. This is called a broth, and it will be used for the further multiplication of micro-organisms and their commercial production. The broth is put in fermenters of the requisite size depending on the amount of broth required to be used in the production of biofertilizer. The broth is aerated continuously by forcing sterile air through a porous stainless steel tube at 10–12 litres of air per hour. The aeration requirement of microbes varies from species to species. When the number of microbes reaches 10^8–10^9 cells/ml, it is considered ready for mixing with the carrier. A large amount of inoculum reduces the time required to reach maximum viable numbers and, therefore, reduces the risk and effects of contamination. Inoculum levels generally vary from 0.1 to 1 percent (sometimes 5 percent). Generally, the process should provide 10^6–10^7 bacteria per millilitre of culture medium at the beginning of fermentation. The fermentation time also varies from 6 to 18 hours depending on species, growth conditions and the initial amount of microbes taken from the mother culture.

Apart from large-capacity steel fermenters, broth can also be fermented/incubated in large conical flasks (2–5 litres), which are mounted and shaken continuously on a rotary shaker for 12–36 hours in order to achieve the same population of microbes as in steel fermenters.

MEASUREMENT OF MICROBIAL GROWTH

Microbial cells are usually counted using a Petroff-Hauser bacterial counter (after placing it under a phase-contrast or dark-field microscope). This counter consists of a thick slide containing one block, which is divided into ten sub-blocks with grooves. The depth of each groove is 0.2 mm. Each sub-block has the capacity to retain a definite number of micro-organisms. Thus, the number of microbes in a block can be determined. It is observed that 1 ml of liquid pure culture of bacterium medium may contain about 5 000 million bacteria.

The growth of any micro-organism (e.g. bacteria) can also be measured by counting the number of colonies developed on Petri dishes. Colony counting is a commonly practised method in microbiological laboratories.

There are many techniques for counting viable cells that are able to divide and form offspring. The usual method is to determine the number of cells in a given sample capable of forming colonies on a suitable agar media. The method involves:

- serial dilution;

- spreading of diluted suspension on plates, and counting of colony-forming units on plates.

Serial dilution

A laminar air-flow chamber is used in order to achieve serial dilution of the broth culture of the strain or biofertilizer sample suspension. For plate counts, the countable range is generally 30–300 cells/ml. To achieve this concentration, the procedure is:

1. Set out 8 tubes, each containing 9 ml of sterile water.
2. Dilute 1 ml of broth culture or biofertilizer sample suspension (1 g of sample in 9 ml of water) in steps (10^{-1} to 10^{-8}) with a sterilized 1-ml serological pipette equipped with a rubber bulb of 1 ml capacity.
3. Suck up broth culture or sample suspension from tube 1 to the 1-ml mark.
4. Immediately expel the broth culture or sample suspension back into the tube with sufficient vigour to effect a thorough mixing.
5. Repeat sucking up and expelling 5 times, and then transfer 1 ml to tube 2.
6. Take a new sterile pipette, attach the rubber bulb, and remove 1 ml to tube 3.
7. Repeat this procedure using a fresh sterile pipette each time until the dilution series is completed.
8. After completion of serial dilution, put an aliquot of the diluted sample on a pour plate, spread plate or drop plate with specified nutrient agar media (as described below). After incubation at 28 ± 2 °C for 3–5 days, each viable cell will give rise to one distinct colony, which is counted and calculated for the number of viable cells per millilitre of suspension. The microbes so grown are taken for further multiplication and testing for their efficiency/quality.

Spreading of diluted suspension on plates and colony counting

Pour plate method

In this method, sterilized molten medium is kept ready in conical flasks placed in a water-bath at a constant temperature of 48 °C. The procedure is:

1. Remove dry sterilized Petri dishes from paper packs and stack them in a laminar air-flow chamber.
2. For each dilution, three Petri dishes will be required. Stack the plates in sets of three each and label them with the help of a glass marker pen.
3. Using a fresh sterile pipette, pour 1 ml of aliquot from the last dilution (say, 10^{-8}) into each of the three Petri dishes.
4. Using the same pipette, pour similar aliquots from the next two dilutions (say, from 10^{-7} and 10^{-6}) into three Petri dishes for each dilution.
5. Pour about same volume of molten medium into each of the plates.
6. Immediately after pouring, move the plates gently in a whirling motion to mix the contents.
7. Allow the medium to solidify, and incubate at 28 ± 2 °C for 3–5 days.
8. Count the colonies after 3–5 days.

9. Multiply the average number of colonies by the dilution factor. If the average number of colonies at 10^{-8} dilution is 60, then the sample had a concentration of $60 \times 10^8 = 6 \times 10^9$ cells/ml.

Spread plate method

Using the same serially diluted samples prepared for the previously described pour plate method, the procedure is:

1. Begin with the 10^{-7} dilution, and deliver 0.1 ml of the sample into each of 4 plates of yeast extract mannitol agar (YEMA) medium previously dried at 37 °C for about 2 hours.
2. Using the same pipette, dispense 0.1-ml samples from the 10^{-6} and 10^{-5} dilutions, in that order.
3. Prepare a glass spreader by bending a 20-cm glass rod of 4 mm diameter to the shape of a hockey stick, dip it into alcohol and hold on flame, then cool the spreader by touching it on the surface of a separate YEMA plate.
4. Lift the cover of each Petri dish just enough to introduce the spreader, and place it in position on the agar surface.
5. Spread the sample evenly over the agar surface, sterilizing and cooling the spreader between samples.
6. Incubate as before.
7. Calculate the number of viable cells as outlined for the pour plate method, adjusting for the smaller volume plated (0.1 ml instead of 1.0 ml). For example, if 60 colonies were counted on a plate inoculated with 0.1 ml of a 10^{-7} dilution, the results should be $60 \times 10 \times 10^7 = 6 \times 10^9$ cells/ml.

Drop plate method

For the drop plate method, the procedure is:

1. Select three-day-old agar plates, which have been dried enough to absorb some moisture, or dry the agar plates in a bacteriological incubator at 37 °C for 2 hours.
2. Take a fixed-volume or variable-volume microlitre pipette and set the volume 30 µl.
3. Sterilize appropriate-sized microtips in a microtip box and keep them ready.
4. Take two Petri dishes containing solidified and dried agar media and divide the bottom of each plate into eight equal parts.
5. Using a microlitre pipette, deliver one drop (30 µl) of diluted suspension in one part.
6. From the last dilution (say, 10^{-8}), deliver four aliquots of 30 µl in each of the four parts of the Petri dish. Use the remaining four parts for the next dilution (say, 10^{-7}). Repeat the process for two further dilutions.
7. Incubate the plates in incubator. In this case, as the area used is very small, observations are to be recorded at the earliest. Otherwise, overlapping of colonies will make counting difficult.

Other methods in brief

Roll-tube method

In this method, the dilutions are mixed with molten agar in a test-tube or bijou bottle, and rolled mechanically while setting, so that the agar forms a film. Aerobes may then be incubated as usual. Anaerobic counts usually require pyrogallol plugs to be inserted in the tube, or some other device for nursing in an anaerobic atmosphere.

Surface-spread method

This is the plating method for aerobes. The agar plate should be dried for 5 minutes at 55 °C or for 45 minutes at 37 °C. The next step is to add 0.2-ml portions from each dilution where the culture is diluted to different levels, say, 1/10, 1/20, and spread evenly over each surface in turn with a wire or a glass spreader in the shape of an L in order to avoid the passage of bacteria under the agar through the plate edge. The disadvantage of this method is that some organisms are taken up by the glass rod or wire, hence, decreasing the count.

Shake-culture method

This method is used to count anaerobes. It involves taking 5 narrow tubes (known as anaerobic or vanilla tubes), adding 0.2 ml of 1/10 dilution of culture and then about 7 ml of molten agar medium (heated at 40 °C or less). While the agar is still molten, the tube is inverted once. Here, some organisms are lost by adhesion to the cotton wool or capsule of the tube. When set, more sterile molten agar is added in order to exclude air. This procedure is not suitable for strongly aerogenic anaerobes. Such organisms (e.g. *Clostridium welchii*) can be counted by a modified roll-tube method. Anaerobes that are not exacting may often be counted on ordinary Petri dishes (as described for the surface-spread method) if these are incubated in an atmosphere of N_2 or H_2 containing anaerobic jar.

Membrane counts

This method involves filtering a known volume of sample through a sterile membrane of a pore size suitable to retain all the required microbes, then transferring the membrane to a nutrient pad, and counting the colonies that grow after incubation. For reasons that are not yet clear, this procedure is not very successful with very exacting anaerobes, but it can be used for aerobes.

Other methods for determining the total number of bacteria

The following methods are also used in order to determine the total number of bacteria (dead or alive):

- Turbidimetry method and nephalometery method: These methods depend on the estimation of turbidity by scattered light from a suspension as well as a transmitted beam. The turbidity is caused by the extent of content of microbes in a given sample. The estimation is done spectrophotometrically.

- Dry-weight method: All the cells harvested from a culture are weighed. This method is not very accurate.
- Cell packing by centrifugation: A sample of cell suspension is centrifuged in a special tube, and the packed cell volume is calculated from the height of the column of solid material.
- Analysis of cellular components such as N, C, H, P, protein, DNA and RNA.

QUALITY CONTROL OF BIOFERTILIZERS

In addition to the counting of viable cells (as detailed above, but described in brief here), the methods for testing the efficiency (as a measure of quality) of different biofertilizers are described below.

Rhizobium

The apparatus required consists of:

- some graduated pipettes – 1 and 10 ml;
- some dilution bottles or flasks;
- some Petri dishes – uniform, flat-bottomed;
- a hot-air oven;
- an autoclave;
- an incubator;
- a hand tally or mechanical counting device;
- a pH meter.

The reagents required are:

- Congo red – 1 percent aqueous solution;
- CREYMA medium.

The procedure is:

1. Put 1.0 g of the biofertilizer sample in a test-tube. Add 9 ml of water and make a suspension. Serially dilute this suspension (as described above). Use the diluted suspension to grow the bacteria by any of the plating techniques described above.
2. Label the plates and incubate at 28 ± 2 °C for 3–5 days for fast-growing Rhizobia and 5–10 days for slow-growing ones.
3. Count the colonies with the aid of a magnifying lens under uniform and properly controlled artificial illumination. Use a colony counter, equipped with a guide plate and rules in square centimetres. Record the total number of colonies with the hand tally.
4. Count all plates, but for the purpose of calculation consider plates showing more than 30 and fewer than 300 colonies per plate. Disregard colonies that absorb Congo red and stand out as reddish colonies. *Rhizobium* stands out as white, translucent, glistening and elevated colonies. Count such colony numbers, and calculate the figure in terms of per millilitre of suspension used for plating. Relate it to the original sample (1 g), taking into account the dilution factor. Also check for being free from contamination at 10^{-6} dilution.
5. Take uncontaminated *Rhizobium* cells and multiply them further for use in nodulation tests in pot culture trials.

TABLE 21
Plant nutrient solution

Composition	g/litre
Potassium chloride	0.0745
Potassium hydrogen phosphate (K_2HPO_4)	0.175
Calcium sulphate ($CaSO_4.2H_2O$)	0.344
Magnesium sulphate ($MgSO_4.7H_2O$)	0.246
Trace elements solution	
Copper sulphate ($CuSO_4.5H_2O$)	0.78
Zinc sulphate ($ZnS0_4.7H_2O$)	2.22
Manganese sulphate ($MnSO_4.4H_2O$)	2.03
Ammonium molybdate [$(NH_4)_6MO_7O_{24}.4H_2O$]	0.01
Boric acid (H_3BO_4)	1.43
Iron solution	
Ferrous sulphate	50.00
Citric acid	50.00

Pot culture test for nodulation and N fixation

A microbiological laboratory normally has a growth room with a growth chamber for carrying out studies/tests through pot culture, etc.

Prepare the nutrient solution by dissolving potassium chloride (0.0745 g), potassium hydrogen phosphate (0.175 g) and magnesium sulphate (0.246 g) in 1 litre of water. To this solution, add 0.5 ml of trace elements solution (respective amounts of micronutrient salts as given in Table 21 are dissolved in 1 litre of water) and 0.5 ml of iron solution (respective amounts are dissolved in 1 litre). In a mortar, grind 0.344 g of calcium sulphate to a fine consistency and add it to the final nutrient solution. Autoclave the nutrient solution at 120 °C for 20 minutes. Keep the pH of the solution at 6.0.

The procedure for pot culture is:

1. Immerse legume seeds in 95 percent alcohol, and wash with chlorine water and then with 0.1 percent mercuric chloride solution for 2–3 minutes. Wash the seeds with sterile water in order to remove the sterilant.
2. Fill glazed pots of 2 kg capacity with soil (2 parts soil and 1 part sand), and autoclave for 2 hours at 120 °C.
3. Inoculate the surface of the sterilized seeds with a water slurry of inoculant taken from a culture packet (biofertilizer sample). Depending on the size of the seeds, 1 ml of inoculant inoculates 15–100 g of seeds.
4. Keep a set of pots sown with non-inoculated seeds as control, another set with inoculated seeds and a third set with ammonium nitrate at 100 kg N/ha. Take four replications of each treatment.
5. Keep the pots in the growth room.
6. Add nutrient solutions to the pots at the start to attain WHC and subsequently with sterile water to keep the soil moist.
7. After 2–3 weeks of growth, thin down the number of plants in each pot to four uniform plants.
8. After 6–8 weeks, harvest the plants separately from each set, and separate the plants carefully from the soil under slow running water. Observe the number, colour and mass of nodules for each treatment.
9. If good effective pink nodulation is obtained in inoculated plants together with absence (or sometimes presence) of stray nodules in controls, and if there is at least a 50-percent increase in the dry matter yield of plants compared

with non-inoculated controls, it may be concluded that the culture is of the required quality. The growth and dry matter yield with ammonium nitrate treatment enables a comparison between the N used through fertilizers and the N fixed through inoculation, i.e. the extent of contribution in terms of N fixation by *Rhizobium*.

Azotobacter

The apparatus required is the same as that for *Rhizobium* (above).

The reagent required is Beijerinckia medium (composition given above).

The procedure is the same as that for *Rhizobium* above.

Azotobacter chroococcum colonies are gummy, raised and often sticky. The pigmentation varies from very light brown to black. Count the number of colonies, observe the cyst formation, and calculate the number per gram of the carrier material.

The uncontaminated cells can then be taken and multiplied further for use in the test for N fixation in pure culture.

Test for nitrogen fixation by pure culture

With pure culture medium, the procedure is:

1. Prepare Beijerinckia medium as per the composition given above.
2. Take *Azotobacter* from a suitable colony. Multiply and use this culture for inoculating the broth for N fixation.
3. For this purpose, put 50-ml aliquots of broth in a 250-ml conical flask, and inoculate with *Azotobacter*. Keep a non-inoculated flask as a control. After 12 days' growth at 28 °C, test the contents of the flask for purity by streaking on fresh medium and concentrating over a water-bath (50–60 °C) to dryness. Wash the dried culture, and take it as a sample. Process the contents of the flask in the non-inoculated controlled series in the same manner.
4. Determine the N in the sample using the Kjeldahl method (Chapter 3). The difference in the N content between the control and the inoculated flasks will indicate the N-fixing capacity of *Azotobacter*.

Azospirillum

The apparatus required is the same as that for *Rhizobium* (above).

The reagent required is N-free bromothymol blue medium (as described above).

The procedure is the same as that for *Rhizobium* (above).

For colony counting, count the tubes or plates that have turned blue in colour after inoculation and ascertain the presence of pellicles in undisturbed medium. Distinct subsurface pellicle formation in the malic acid medium is the confirmatory test of the presence of pure *Azospirillum* in the inoculant. However, the test for N fixation by pure culture as described for *Azotobacter* (above) is also valid for testing the purity of *Azospirillum*. Count the colony numbers, and calculate the number per gram of the carrier material.

Phosphate-solubilizing bacteria

The apparatus required is the same as that for *Rhizobium* (above).

The reagent required is Pikovskaya medium as per composition given under media preparation (above).

The procedure is the same as that for *Rhizobium* (above).

For colony counting, count the total number of colonies on the plates including colonies with a solubilization zone with the help of a colony counter. Measure the diameter of the solubilization zone. The minimum acceptable zone is 10 mm in diameter. The greater the solubilization zone is, the higher is the efficiency of the bacteria.

COMMERCIAL PRODUCTION OF BIOFERTILIZERS

In addition to quality control of biofertilizers/bioinoculants, service laboratories may be able to produce quality biofertilizers on a commercially viable scale.

Rhizobium

The types and composition of various media suitable for growing *Rhizobium* species have been described above. For commercial production, a liquid medium (without agar), such as Norris and Date (1976), is used.

Non-sterile production system

This system uses an unsterilized carrier.

The procedure is:

1. Prepare liquid media in conical flasks as described above.
2. Sterilize the media in an autoclave.
3. Add pure culture at 10 ml/litre of sterilized medium in each flask. This is called broth.
4. Incubate the flasks on a rotary shaker at 28–30 °C for 36–48 hours. There should be a population of 10^8–10^9 cells/ml at the end of the incubation. Broth can also be incubated in a fermenter of suitable size.
5. Neutralize the carrier (lignite) with a suitable chemical (lime), and add gum acacia at 4 g per 100 g of carrier.
6. Mix the broth culture with the carrier to attain a moisture content of about 30–40 percent, and incubate at 28 °C for 24 hours. Leave for curing for 2–3 days.
7. Dispense the cured broth-mixed carrier in polyethylene pouches. Pouches of 250 g, 500 g or 1 kg are prepared to meet the requirement of different quantities of seed to be treated for sowing in a given area.
8. Seal the pouches by heat sealer.
9. Put the pouches in printed polyethylene packets.
10. Store the packets at less than 20 °C until dispatch.

Sterile production system

This system uses a sterile carrier.

The procedure is:

1. Prepare liquid media in conical flasks.
2. Sterilize the medium in an autoclave.
3. Add pure culture at 10 ml/litre of sterilized media in each flask. This is called broth.
4. Incubate the flasks on a rotary shaker at 28–30 °C for 36–48 hours. There should be a population of 10^8–10^9 cells/ml at the end of incubation. Broth can also be incubated in a fermenter of suitable size.
5. Neutralize the carrier with a suitable chemical (lime), and add gum acacia at 4 g per 100 g of carrier.
6. Put the carrier in polypropylene bags, and autoclave for 4–6 hours.
7. Seal the pouches after autoclaving and keep them for 2 days before inoculation.
8. Wipe the pouches with alcohol.
9. Dispense the desired broth culture aseptically with an automatic dispenser by making holes in the polypropylene pouches or packets.
10. Seal the hole immediately with tape.
11. Blend the packets only manually to ensure that broth mixes with the carrier adequately (the sealed pouches/packets have to be manually manipulated to ensure mixing).
12. Incubate the pouches for further multiplication of microbes by keeping the pouches in the incubator at 28 °C for 24 hours. Leave for 2–3 days for curing.
13. Put the pouches in printed polyethylene packets.
14. Store the packets at less than 20 °C until dispatch.

Table 22 lists the quantities of chemicals required for the production of 25, 50 and 100 tonnes of *Rhizobium* biofertilizer.

TABLE 22
Chemicals required for the production of *Rhizobium* biofertilizer

Chemicals required	Biofertilizer (30–40% moisture)		
	25 tonnes	*50 tonnes*	*100 tonnes*
		(kg)	
Mannitol	87.50	175.00	350.00
Yeast extract	8.50	17.50	34.80
K_2HPO_4	4.50	8.50	17.50
$MgSO_4.7H_2O$	7.00	14.00	28.00
NaCl	1.75	3.50	7.00
$FeCl_3.6H_2O$	0.10	0.20	0.50

Note: Calculated on the basis of Norris and Date (1976) medium.

Azotobacter

The production process is similar to that for *Rhizobium* production. However, *Azotobacter*-specific broth is prepared. This is the same medium as that for laboratory use (Jensen's or Ashby's media) except that agar is not added in the preparation of broth to be used for carrier-based commercial production. Table 23 lists the quantities of chemicals required for the production of 25, 50 and 100 tonnes of *Azotobacter* biofertilizer.

Azospirillum

The procedure is same as for *Rhizobium* and *Azotobacter* (above). The broth is prepared in N-free bromothymol blue medium (excluding the use of agar) and/or Okon's modified liquid medium (excluding bromothymol blue because bromothymol blue is only for identification/confirmation – once it is confirmed, there is no need to add it again).

Table 24 lists the quantities of chemicals/reagents required for the production of 25, 50 and 100 tonnes of *Azospirillum*.

Phosphate-solubilizing micro-organisms

Isolated and purified culture of PSM is further multiplied by using Pikovskaya medium for commercial production. The same procedure as followed for production of *Rhizobium*, *Azotobacter* and *Azospirillum* (above) can be adopted. As in the other cases, agar is not used in the medium. Liquid broth and charcoal/lignite as carrier are used by maintaining a moisture content of the product at about 30–40 percent.

Table 25 lists the chemicals/reagents required for the production of 25, 50 and 100 tonnes of PSMs.

TABLE 23
Chemicals required for the production of *Azotobacter* biofertilizer

Chemicals	Biofertilizer (30–40% moisture)		
	25 tonnes	*50 tonnes*	*100 tonnes*
		(kg)	
Sucrose	150.00	300.00	600.00
K_2HPO_4	7.50	15.00	30.00
$MgSO_4.7H_2O$	3.75	7.50	15.00
NaCl	3.75	7.50	15.00
$FeSO_4$	0.75	1.50	3.00
$CaCO_3$	15.00	30.00	60.00

Note: Calculated on the basis of Jensen's medium.

TABLE 24
Chemicals required for the production of *Azospirillum* biofertilizer

Chemicals	Biofertilizer (30–40% moisture)		
	25 tonnes	*50 tonnes*	*100 tonnes*
		(kg)	
K_2HPO_4	45.00	90.00	180.00
KH_2PO_4	30.00	60.00	120.00
$MgSO_4$	1.50	3.00	6.00
NaCl	0.75	1.50	3.00
$CaCl_2$	0.15	0.30	0.60
NH_4Cl	7.50	15.00	30.00
Malic acid	37.50	75.00	150.00
NaOH	22.50	45.00	90.00
Yeast extract	0.40	0.80	1.50
Na_2MoO_4	0.015	0.03	0.06
$MnSO_4$	0.01	0.02	0.04
H_3BO_4	0.01	0.02	0.04
$Cu(NO_3)_2$	0.005	0.01	0.02
$ZnSO_4$	0.015	0.03	0.06
$FeCl_3$	0.015	0.03	0.06
Bromothymol blue (0.5% alcoholic)	15 litres	30 litres	60 litres

Note: Calculated on the basis of Okon's medium.

TABLE 25
Chemicals required for the production of PSMs

Chemicals	Biofertilizers (30–40% moisture)		
	25 tonnes	*50 tonnes*	*100 tonnes*
		(kg)	
$Ca_3(PO_4)_2$	37.50	75.00	150.00
Sucrose	75.00	150.00	300.00
$(NH_4)_2SO_4$	3.75	7.50	15.00
NaCl	1.50	3.00	6.00
$MgSO_4.7H_2O$	0.75	1.50	3.00
KCl	1.50	3.00	6.00
Yeast extract	3.75	7.50	15.00
$MnSO_4$	0.01	0.02	0.04
$FeSO_4$	0.01	0.02	0.04

Note: Calculated on the basis of Pikovskaya medium.

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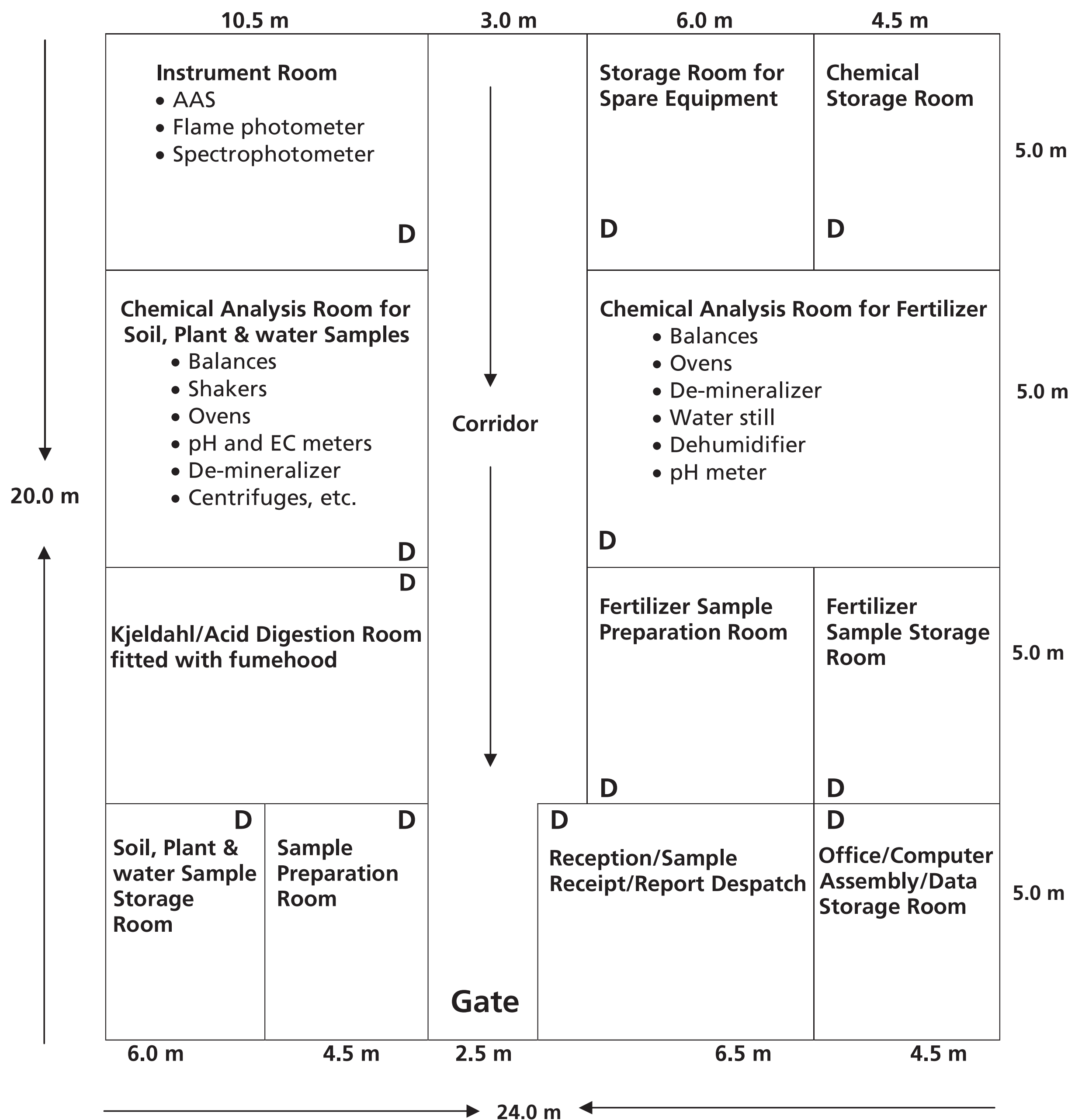
Annex 1

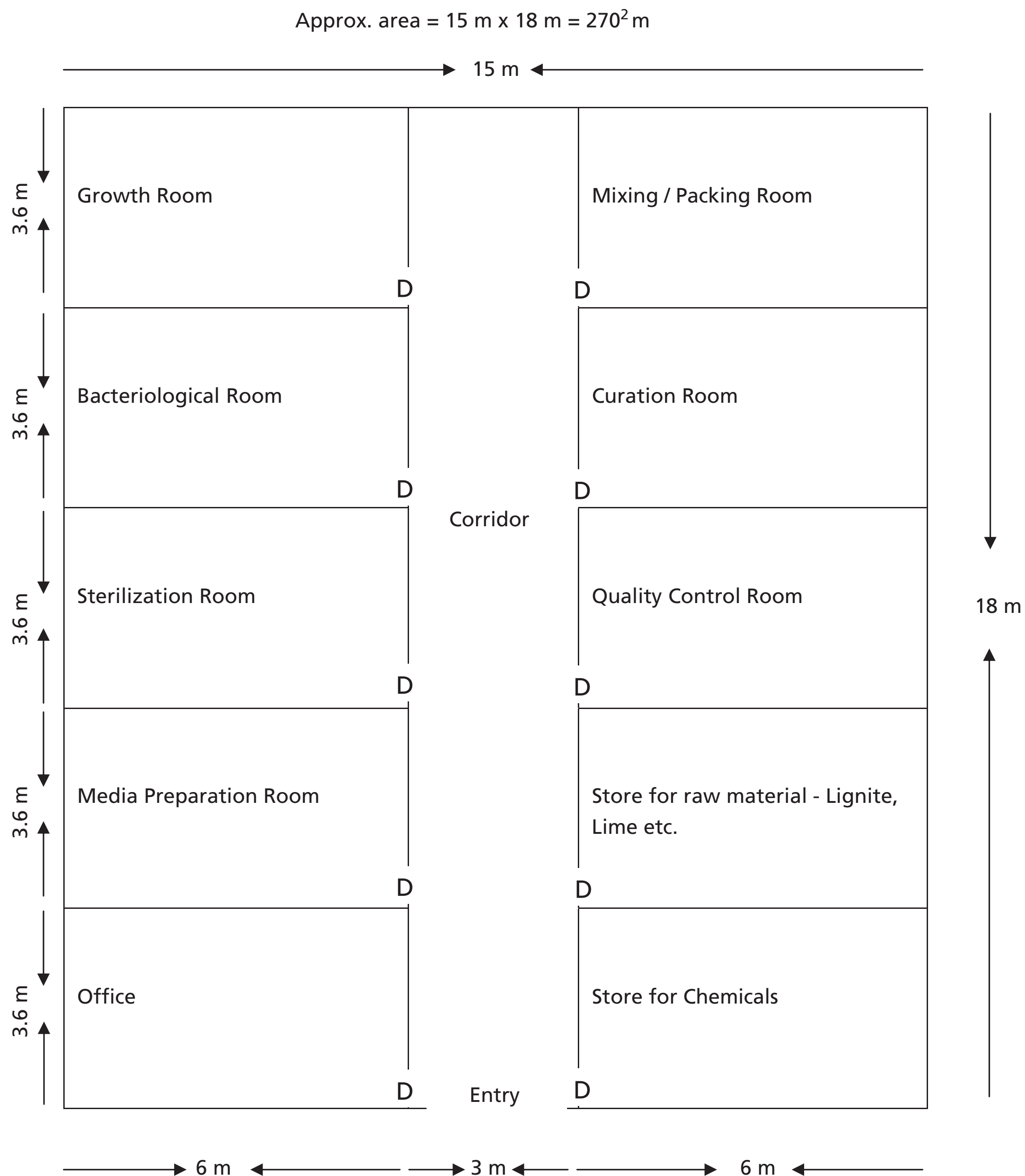
Floor plan of a soil, plant, water and fertilizer analysis laboratory

FIGURE A1.1
Laboratory floor plan for soil, plant, water and fertilizer analysis



Annex 2
Floor plan of a biofertilizer laboratory and production unit

FIGURE A2.1
Floor plan of a biofertilizer laboratory and production unit



Annex 3

Items required for a soil, plant and water analysis laboratory

Tables A3.1–A3.3 list the equipment, chemicals and glassware required for a laboratory with a capacity to analyse 10 000 soil samples, 1 000 plant samples and 500 water samples annually.

TABLE A3.1
Equipment

Serial No.	Name/specification/description	No.
1.	Analytical / semi-micro balance Capacity = 300 g Resolution = 0.1 mg Pan size = 100 mm	2 (1 of each type)
2.	Two-pan balance Capacity = 500 g Resolution = 0.2%	1
3.	pH meter: range 0–14 pH with accuracy of ±0.05 pH, complete with combination electrode, mains operated; to work on 220 V, 50 cycles	2
4.	Conductivity bridge: single range 0–15 mS/cm directly calibrated with temperature compensation and cell constant adjuster, complete with pipette-type conductivity cell having platinum electrodes duly coated with platinum black and having a cell constant of 1.00±0.01%; mains operated with electronic "eye" null indicator; to work on 220 V, 50 cycles	1
5.	Photoelectric colorimeter: with dual-barrier-type matched photocells with sensitive galvanometer for null adjustment and logarithmically calibrated potentiometer to read directly optical density on dial, complete with optical glass filters for maximum transmission at 420, 540 and 660 nm wavelengths; mains operated; to work on 220 V, 50 cycles Or spectrophotometer	1
6(i).	Shaking machine: reciprocating type, variable speed of 70–300 strokes per minute, with box-type platform carrier (size: 79 cm long × 43 cm wide × 8 cm high) fitted with heavy-duty electric motor for continuous operation and built-in 0–60 minutes timeswitch; to work on 220 V, 50 cycles	1
6(ii).	Water-bath shaker with 50 cm × 38 cm water-bath size mounted on the shaking machine as 6(i)	1
7.	Centrifuge: clinical type with head to take 12 tubes of 15 ml capacity, complete with metal shields, rubber cushions and 15 ml centrifuge polyethylene tubes; to work on 220 V, 50 cycles	1
8.	Voltage stabilizers (constant voltage transformers): input 170–250 V, AC output 220 V Capacity: 1, 2 & 5 kW	1 each
9.	Voltage stabilizers (constant voltage transformers): Input 170–250 V, AC output 220 + 5 V	2
10.	Pressure vacuum pump: to deliver 0.04 m^3 of air per minute at a maximum pressure of 1.1 kg/cm^2 and create vacuum of 28 ° mercury column, complete with pressure gauge and ballast; to work on 220 V, 50 cycles (SI unit)	2

Serial No.	Name/specification/description	No.
11.	Automatic pipetting machine: Brewer-type, adjustable volume and frequency of cycling, to deliver up to 50 ml solution; syringe, plunger cylinder, valves and intake and delivery tubes made of neutral hard glass or resistant plastic material; to work on 220 V, 50 cycles	2
12.	Demineralizer plant: For obtaining deionized water, regenerating type, separate or mixed-bed resin columns treated water to have pH 6.8–7.0, portable model, capacity – 75 litres of deionized silica free water per hour	1
13.	Oven: laboratory model, made of stainless steel inside and outside, maximum temperature 180 °C, thermostatically controlled, ±1.0 °C accuracy, inside chamber – 30 cm × 30 cm × 30 cm	1
14.	Trolleys (push carts) made of tubular frame mounted on rubber casters, with mild steel top; size 60 cm × 75 cm and with a height of 90 cm	4
15.	Flask stand: To hold eleven 100 ml conical flasks, with adjustable base and collar – total length of stand 75 cm, width 8 cm, overall height 12 cm, distance between flasks 6.5 cm to fit multiple dispensing equipment (wood)	10
16.	Funnel stand: To hold eleven 5–7 cm diameter glass funnels, to correspond to flask stand	10
17(i).	Beaker stand: To hold eleven 50 ml beakers – to fit multiple dispensing equipment (wood)	10
17(ii).	Burette stand with clamps	4
18.	Test-tube stand: To hold 11 test-tubes of 25 mm × 150 mm, distance between test-tubes 6.5 cm, overall height 16 cm (wood)	10
19.	Multiple dispensing equipment: with 11 units each 50 ml, to fit 75 cm tray, distance between flasks from centre to centre 6.5 cm, width of tray 8 cm, distance from centre of outer flasks and end of tray 4.5 cm, overall height of tray 12 cm	2
20.	Multiple dispensing equipment: with 11 units each 25 ml, to fit 75 cm tray, distance between flasks from centre to centre 6.5 cm, width of tray 8 cm, distance from centre of outer flasks and end of tray 4.5 cm, overall height of tray 12 cm	2
21.	Multiple dispensing equipment: With 11 units each 20 ml, to fit 75 cm tray, distance between flasks from centre to centre 6.5 cm, width of tray 8 cm, distance from centre of outer flasks and end of tray 4.5 cm overall, height of tray 12 cm	2
22.	Washing assembly: for washing glassware used with multiple dispensing equipment, pipe and jet made of PVC, combined unit for both tap-water washing and distilled water rinsing, complete with brackets for mounting in sink	1
23.	Scoops: for soil sampling, made of brass with wooden handle. A set of 5 scoops to measure 1 , 2.5 , 5.0 , 10 and 12.5 g of soil	2
24.	Mortar and pestle: heavy cast-iron mortar or porcelain mortar, glazed outside only, size 160 mm diameter with wooden rubber-tipped pestle	2
25(i).	Sieves: 20 cm in diameter, 5 cm high with 2 mm round holes, preferably made of stainless steel, complete with cover lid and receiver pan	3
25(ii).	1 mm sieve with similar specifications	1
26.	Trays: for drying soil samples, made of aluminium sheet, 22 cm × 22 cm × 8 cm	100
27.	Hotplate: rectangular 45 cm × 60 cm with three positions: low, medium and high, heavy-duty rotary switch, 1 kW to work on 220 V, 50 cycles	2
28.	Kjeldahl assembly, both as digestion and distillation set, having a capacity to hold 6 round-bottomed flask of 300 ml capacity each complete with condenser and connecting tubes Heating capacity of 500 W of each heater	1
29.	Fumehood – digestion chamber to hold Kjeldahl assembly of 6 sets	1
30.	Atomic absorption spectrophotometer: double-beam with spare hollow cathode lamps for zinc, copper, manganese, iron, boron and molybdenum	1
31.	Muffle furnace with temperature 1 000 °C ± 5 °C, size of furnace 10 cm × 15 cm	1

TABLE A3.2
Chemicals

Chemical / item	Estimated annual requirement
Acetic acid (glacial)	10 litres
Activated charcoal – P-free	10 kg
Ammonium ferrous sulphate (CP)	10 kg
Ammonia solution (CP)	10 litres
Ammonium acetate (CP)	40 kg
Ammonium bicarbamate (AR)	1 kg
Ammonium metavanadate (AR)	1 kg
Ammonium molybdate (AR)	3 kg
Ammonium vanadate (AR)	500 g
Ascorbic acid	100 g
Barium chloride (CP and AR)	1 kg each
Boric acid (CP)	10 kg
Bromocresol green	500 g
Buffer solutions (pH 4.0, 7.0 & 9.2)	2 bottles each
Calcium acetate (CP)	500 g
Calcium carbonate (CP)	0.5 kg
Calcium chloride (CP)	0.5 kg
Calcium sulphate (CP)	500 g
Copper sulphate (AR)	500 g
Copper sulphate (CP)	1 kg
Diethylene triamine pentaacetic acid	2 kg
Digestion mixture containing potassium sulphate and copper sulphate	10 kg
Diphenyl amine indicator	100 g
EDTA – disodium salt	1 kg
Eriochrome black – T	50 g
Ethyl alcohol	5 litres
Ferrous sulphate (AR)	500 g
Ferrous sulphate (CP)	500 g
Filter paper, No. 1 (460 mm × 570 mm)	2 500 sheets
Filter paper, 110 mm, No. 40	5 packets
Filter paper, 110 mm, No. 42	5 packets
Gum acacia	0.5 kg
Hydrochloric acid (CP)	100 litres
Hydrogen peroxide	5 litres
Hydroxylamine hydrochloride	1 kg
Magnesium chloride (AR)	500 g
Manganese sulphate (AR)	500 g
Manganese chloride (CP)	1 kg

Chemical / item	Estimated annual requirement
Methyl orange	25 g
Methyl red	25 g
Methylene blue	50 g
Monocalcium phosphate (AR)	500 g
Mureoxide indicator	100 g
Nitric acid (CP)	10 litres
Nitric acid (AR)	10 litres
Nitrophenol (CP)	500 g
Paranitrophenol (CP)	1 kg
Perchloric acid (CP)	10 litres
pH indicator papers (full pH range)	10 books
Phenolphthalein indicator	100 g
Phosphoric acid (LR)	5 litres
Potassium dihydrogen phosphate (KH_2PO_4) (AR)	500 g
Potassium chloride (AR)	2 kg
Potassium chromate (CP)	500 g
Potassium dichromate (AR)	2 kg
Potassium dihydrogen orthophosphate	500 g
Potassium hydrogen phthalate (AR)	500 g
Potassium permanganate (CP)	2 kg
Potassium sulphate (AR)	1 kg
Salicylic acid (CP)	1 kg
Silver sulphate	1 kg
Sodium bicarbonate (LR)	30 kg
Sodium carbonate (AR)	500 g
Sodium carbonate (CP)	1 kg
Sodium cyanide	100 g
Sodium diethyl dithiocarbamate	500 g
Sodium fluoride (CP)	500 g
Sodium hydroxide (CP)	50 kg
Sodium thiosulphate (CP)	500 g
Stannous chloride (AR)	500 g
Sucrose (AR)	500 g
Sucrose (CP)	1 kg
Sulphuric acid (conc./CP)	50 litres
Toluene	500 ml
Tri-ethanol amine granule (CP)	500 g
Tri-ethanol amine	500 ml
Universal indicator	100 ml
Zinc sulphate (AR)	500 g

TABLE A3.3
Glassware

Serial No.	Item	Size/specification	Quantity (No.)
1.	Bottle (polyethylene)	20 litres	5
2.	Bottle (polyethylene)	10 litres	5
3.	Bottle (glass) for reagents with glass stoppers	Glass-stoppered	
		125 ml	10
		250 ml	10
		500 ml	20
		1 000 ml	5
		2 000 ml	5
4.	Bottle (glass), amber	250 ml	5
		500 ml	5
5.	Bottle (polyethylene) – wash bottle	250 ml	6
		500 ml	6
6. (i)	Burettes fitted with screw-thread stopcocks		
	Graduation interval (ml)		
	0.05	10 ml	2
	0.05	25 ml	2
	0.1	10 ml	2
	0.1	25 ml	2
6. (ii)	Burette (automatic)		
	(mounted on reservoir)		
	Graduation interval (ml)		
	0.1	25 ml	2
	0.1	50 ml	2
7.	Cylinder (glass) graduated with an interval of:		
	0.5 ml	10 ml	2
	1 ml	25 ml	2
	2 ml	50 ml	2
	2 ml	100 ml	2
	5 ml	500 ml	2
8.	Crucible (silica)	30 ml	10
9.	Desiccator with approx. I.D. of ground flange as 200 mm		2
10.	Dishes, evaporating flat-bottom with pour out, having outer diameter as 150 mm and height as 80 mm		20
11.	Water distilling unit, mounted with borosilicate condenser, with a capacity (output) to distil 2.5 litre/hour		2
12.	Flask distilling/Kjeldahl, round-bottom, long-neck		
	Capacity — O.D. × height (mm)		
	100 ml — 64 x 210		12
	250 ml — 85 x 226		12
13.	Flask (conical)		
	100 ml cap., 64 × 105 mm (O.D. × height) with approx. neck O.D. as 25 mm		50
	250 ml cap., 85 × 140 mm (O.D. × height) with app. neck O.D. as 34 mm		50

Serial No.	Item	Size/specification	Quantity (No.)
	500 ml cap., 104 × 180 mm (O.D. × height) with app. neck O.D. as 34 mm		10
	1 000 ml cap., 131 × 225 mm (O.D. × height) with app. neck O.D. as 34 mm		10
14.	Flask (volumetric)		
	Capacity (ml)	Tolerance (± ml)	
	25	0.04	50
	50	0.06	50
	100	0.10	100
	250	0.15	25
	500	0.25	25
	1000	0.40	10
15.	Funnel, plain, 60-degree angle		
	Diameter		
	50 mm		20
	65 mm		20
	75 mm		20
	100 mm		20
16.	Pipette (measuring)		
	Capacity (ml)	Graduation interval (ml) / Tolerance (± ml)	
	1.0	0.1 / 0.006	2
	2.0	0.1 / 0.01	2
	5.0	0.1 / 0.05	2
	10.0	0.1 / 0.05	5
	25.0	0.2 / 0.1	5
	50.0	0.5 / 0.1	2
17.	Porcelain dish – 100 and 150 ml		6 each
18.	Test-tube		
	App. O.D. × height (mm)		
	12 × 10		60
	15 × 125		60
	18 × 150		60
19.	Watch glass		
	App. diameter (mm)		
	100		60
	200		60
20.	Rubber stopper		
	15, 18, 20, 25 & 30 mm diameter		12 each
21.	Spatula (stainless) with wooden handle, blade length 100 mm		12
22.	Wire gauge with asbestos centre 150 × 150 mm		20

Notes:
Twenty percent of glassware may need annual replacement because of breakage or changed requirement.
O.D. = outer diameter.
I.D. = internal diameter.
When estimating boron, boron-free glassware must be used. Commonly required glassware includes beakers, flasks, pipettes, funnels and water distilling sets. Generally, marketed glassware is made of borosilicates, which contains traces of boron, hence, not suitable for boron estimation.

Annex 4

Items required for a fertilizer testing laboratory

Tables A4.1 and A4.2 list the equipment, chemicals and glassware required for a fertilizer testing laboratory with a capacity to analyse 2 000 samples (1 000 mineral fertilizer samples and 500 organic fertilizer samples) annually. The glassware requirements are similar in terms of number and type to those for the soil testing laboratory (Annex 3).

TABLE A4.1
Equipment

Name of equipment / specification (details in Annex 3)	No.
Analytical balance (semi-micro) with 0.1 mg resolution	1
Micro balance with 0.01 mg resolution	1
Two-pan balance, 500 g capacity	1
pH meter	1
Spectrophotometer	1
Shaking machine	1
Water-bath shaker	1
Centrifuge	1
Atomic absorption spectrophotometer	1
Voltage stabilizer (1, 2 & 5 kW)	1 each
Pressure vacuum pump	1
Demineralizer plant	1
Oven	1
Hotplate	2
Kjeldhal digestion and distillation unit to hold 6 flasks each	1 each
Fumehood	1
Muffle furnace	1
Automatic pipetting machine	1
Trolleys	4
Flask stand	4
Beaker stand	4
Test-tube stand	4
Pestle and mortar	2
Sieves: 0.25, 0.5, 1, 2 & 4 mm	2 sets each
Trays	6

TABLE A4.2
Chemicals

Chemical/grade	Estimated annual requirement
Acetic acid (glacial) (AR)	1 litre
Ammonium hydroxide (CP)	10 litres
Ammonium metavanadate (AR)	1 kg
Ammonium molybdate (AR)	1 kg
Ammonium oxalate (CP)	2 kg
Ammonium vanadate (CP)	500 g
Barium chloride (AR)	500 g
Benzalkonium chloride (AR)	1 kg
Boric acid (CP)	5 kg
Bromocresol green	100 g
Buffer solution (pH 4, 7 & 9.2)	500 ml each
Citric acid (CP)	2 kg
Clayton yellow	100 g
Copper sulphate (AR)	500 g
Copper sulphate (CP)	5 kg
Devarda's alloy	1 kg
Diphenylamine indicator	100 g
Ferrous sulphate (AR)	500 g
Filter paper	
No. 1 (460 mm × 570 mm)	1 000 sheets
No. 12 (11 cm diameter)	10 packets
No. 44 (11 cm diameter)	20 packets
No. 42 (11 cm diameter)	20 packets
Formaldehyde (CP)	5 litres
Hydrochloride acid (AR)	5 litres
Hydrochloride acid (CP)	20 litres
Magnesium nitrate (CP)	2 kg
Magnesium oxide (CP)	2 kg
Manganese sulphate (AR)	500 g
Methyl orange indicator	100 g
Methyl red indicator	100 g
Monocalcium phosphate (AR)	500 g
Mureoxide indicator	100 g
Nitric acid (AR)	5 litres
Nitric acid (CP)	20 litres
Perchloric acid (CP)	10 litres
Phenolphthalein indicator	100 g
Phosphoric acid (CP)	10 litres
Potassium chloride (AR)	1 kg
Potassium chloride (CP)	2 kg

Chemical/grade	Estimated annual requirement
Potassium chromate (CP)	500 g
Potassium dichromate (CP)	2 kg
Potassium dichromate (AR)	500 g
Potassium ferricyanide (AR)	500 g
Potassium ferrocyanide (AR)	500 g
Potassium hydrogen phosphate (AR)	500 g
Potassium hydrogen phthalate (AR)	1 kg
Potassium permanganate (CP)	1 kg
Potassium sulphate (CP)	5 kg
Quinolinium phosphomolybdate	1 kg
Salicylic acid (CP)	1 kg
Sodium chloride (CP)	1 kg
Sodium cyanide	100 g
Sodium hydroxide (CP)	50 kg
Sodium molybdate (AR)	2 kg
Sodium nitrate (AR)	1 kg
Sodium oxalate (CP)	1 kg
Sodium tetraphenyl boron (AR)	2 kg
Sodium thiosulphate (CP)	500 g
Sucrose (AR)	500 g
Sulphuric acid (AR)	5 litres
Sulphuric acid (CP)	20 litres
Universal indicator	100 ml
Zinc granules (AR)	500 g
Zinc sulphate (AR)	500 g

Annex 5
Items required for a microbiological laboratory

Tables A5.1–A5.3 list the equipment, chemicals and glassware required for a microbiological laboratory with a capacity to analyse 1 000 samples and produce 25–100 tonnes of biofertilizer per year.

TABLE A5.1
Equipment

Serial Number	Item	No.
1.	Binocular microscope for phase-contrast interchangeable inclined binocular body, rotatable through 360 °, high-quality eyepiece in pairs of 7×, 10×, 15× and parfocal, achromatic objectives of 4×, 10×, 40× and 100×.	1 set
2.	Trinocular microscope with CCD specification, nano CAM 2:	1 set
	- CCD chip: 0.8 cm	
	- video system: PAL	
	- resolution: 460 lines horizontal	
	- minimum luminance: 2.5 lux.	
	- autoshutter: 1/50 – 1/30 000	
	- video signal: composite	
3.	Autoclave, bench-top:	1
	- chamber capacity: 30 litres	
	- chamber dimensions (h × w × d): 265 × 225 × 450 mm	
	- bench space required (w × d): 483 × 610 mm	
	- mass: 110 kg	
	- power: 2 600 W	
	- temperature range: 100–140 °C	
	- pressure range: 0–3 kg/cm^2	
	- power supply: 220/240 V, 50 Hz	
	Accessories:	
	- basket, stainless steel mesh of 40 × 215 × 155 mm	
4.	Centrifuge:	1
	- maximum speed: 14 000 rpm	
	- maximum rpm: 20 800	
	- maximum load: 4 × 250 ml	
	- power supply: 230 V, 50–60 Hz	
	- maximum power required: 1 100 W	

Serial Number	Item	No.
	- dimensions (w × d × h): 700 × 608 × 345 mm	
	- weight: 99 kg	
	Accessories:	
	- swing-out rotor 4 × 250 ml including four 250 ml rectangular buckets	
	- aerosol tight cups for 250 ml buckets	
	- adapters for 250 ml buckets	
	- 4 buckets for swing-out rotor each holding 4 microtitre plates	
	- centrifuge tube of 5 ml	
	- centrifuge tube of 15 ml	
5.	Digital bacteriological and cell culture incubator:	1
	- capacity: 400 litres	
	- internal dimensions (h × w × d): 100 × 80 × 50 cm	
	- external dimensions (h × w × d): 130 × 114 × 75 cm	
	- shelf positions: 10	
	- power: 1 000 W	
	- weight: 160 kg	
	Accessories:	
	- shelves	
6.	CO_2 incubator for anaerobic cell and tissue culture:	1
	- capacity: 136 litres	
	- internal dimensions (h × w × d): 62 × 44 × 50 cm	
	- external dimensions (h × w × d): 100 × 60 × 60 cm	
	- shelf positions: 6	
	- power: 550 W	
	- weight: 70 kg	
7.	Drying and sterilizing oven:	1
	- capacity: 150 litres	
	- internal dimensions (h × w × d): 50 × 60 × 50 cm	
	- external dimensions (h × w × d): 70 × 95 × 68 cm	
	- shelf positions: 8	
	- power: 2000 W	
	- weight: 68 kg	
	Accessories:	
	- guides	
	- shelves	
8.	Laminar air flow:	1
	Cabinet: horizontal type with a workspace of 120 × 60 × 60 cm provided with pre-filters and HEPA filters with a capacity to retain particles of 0.2–0.3 micron (0.0002 – 0.0003 mm) from the air. Workhood covered with perspex sheets and provided with fluorescent illumination system and UV germicide tubes.	
9.	Analytical / semi-micro balance:	1
	- capacity: 310 g	
	- resolution: 0.1 mg	

Serial Number	Item	No.
	- pan size: 90 mm diameter	
10.	Multifunctional precision balance:	1
	- capacity: 410 g	
	- resolution: 0.001 g (1 mg)	
	- pan size: 120 mm	
11.	Thermostatic water-bath/circulator:	1
	- temperature range: -15 °C to +150 °C	
	- stability: 0.004 °C	
	- uniformity: ±0.001 °C	
	- tank capacity: 38 litres	
	- tank dimensions (l × w × d): 690 × 300 × 200 mm	
	- top opening (l × w): 580 × 300 mm	
	- liquid depth (min./max.): 120/180 mm	
	- overall (l × w): 720 × 325 mm	
	- height to tank rim: 225 mm	
	- overall height: 320 mm	
	- pump, maximum head: 1.7 m	
	- maximum flow of water: 8.5 litres/minute, with adjustable cutout	
	- head power: 1.4 kW	
	- total consumption: 1.5 kW	
	Accessories:	
	- lid for bath: 38 litres	
	- raised shelves	
	- lid hinge	
12.	Micropipettes:	5 each
	- adjustable: 0.5–10 µl (microlitres)	
	- adjustable: 10–50 µl	
	- adjustable: 50–200 µl	
	- adjustable: 200–1 000 µl	
	Pipette tips:	
	- tips: 5–200 µl	
	- tips: 201–1 000 µl	
	- adapter volume: 0.5–10 µl	
	- adapter volume: 30–300 µl	
13.	Stand for pipettes	3
14.	Hotplate, circular:	1
	General purpose with thermocouple control from ambient to 350 °C, epoxy-finished case is acid and impact resistant. The 600 W silumin hotplate diameter is 135 mm, 220 V, 50 Hz	
15.	Microscope slide box: polished, wooden box with slots to hold up to 100 slides	2
16.	Dissecting instruments kit:	2
	Dissecting kit in a wooden case. Contains: 1 fine-point needle, 1 needle (lancet-type), 1 set of blunt-tip forceps, 1 set of fine-point forceps, 1 spatula handle No. 4 with 3 sterile blades (Nos. 20, 22 and 23) and 1 pair of scissors	

Serial Number	Item	No.
17.	Jar, anaerobic, 2.5 litres	2
18.	Colony counter:	1
	For use with standard Petri dishes up to 100 mm in diameter; the counter has circular glare-free illumination and a choice of dark or light background, solid-state electronics with pressure-operated counting adjustable sensitivity and audible indication of count. Digital readout (to 999) and reset button mounted on the top panel, main switch and sensitivity adjustment at rear. For use on 220/240 V, 50 Hz	
19.	Mortar and pestle: porcelain, non-glazed with spout	3 sets
20.	Water distilling unit, mounted with borosilicate condenser with a capacity (output) to distil 2.5 litres/hour	2

TABLE A5.2
Chemicals – for analysing 1 000 samples, CP-grade

Chemical/item	Estimated annual requirement
Agar powder	2 kg
Ammonium chloride	1 kg
Ammonium oxalate $(COONH_2)_2.H_2O$	500 g
Aniline blue (water-soluble) for microscopical staining	25 g
Boric acid	500 g
Bromothymol blue	100 ml
Calcium carbonate	5 kg
Calcium chloride	1 kg
Calcium sulphate precipitated $(CaSO_4.2H_2O)$	500 g
Congo red	100 g
Congo red for microscopical staining	20 × 25 g
Copper sulphate, anhydrous $(CuSO_4)$	500 g
Dipotassium hydrogen orthophosphate heptahydrate, anhydrous (K_2HPO_4)	500 g
Ethanol 96% V/V (C_2H_5OH)	4 × 500 ml
Ethanol 99.7–100% V/V (absolute) (C_2H_5OH)	500 ml
Ethylenediamine tetraacetic acid (ferric monosodium salt) $[(CH_2N(CH_2.COOH)_2)_2FeNa]$	250 g
Ferric chloride	500 g
Fuchsin (acid) for microscopical staining	25 g
Fuchsin (basic) for microscopical staining	5 × 100 g
Glucose	2 × 500 g
Iodine resublimed	500 g
Lactophenol	25 g
Lactophenol blue	25 g
Magnesium sulphate - dried $(MgSO_4.H_2O)$	500 g
Magnesium sulphate heptahydrate - molecular biology grade $(MgSO_4.7H_2O)$	500 g
Malic acid	2 × 500 g
Manganese chloride $(MnCl_2.4H_2O)$	500 g
Mannitol	2 × 500 g
Molybdenum trioxide - AR (MoO_3)	100 g
Orthoboric acid powder (H_3BO_3)	500 g
Peptone	2 × 500 g
Phenol (redistilled molecular biology grade) (C_6H_5OH)	500 g
Potassium dihydrogen orthophosphate (KH_2PO_4)	500 g
Potassium hydroxide	500 g
Potassium iodide (KI)	500 g
Potassium sulphate (K_2SO_4)	500 g
Saframin O for microscopical staining	10 × 25 g
Sodium chloride (NaCl)	20 × 25 g
Sodium molybdate	500 g
Sucrose	2 × 500 g
Yeast extract	500 g
Zinc sulphate $(ZnSO_4.7H_2O)$	500 g

TABLE A5.3
Glassware

Item	No.
Pipette, disposable, pre-plugged with non-absorbent cotton wool including 8 PVC teats	1 Pack of 1 000
Beaker 2 000 ml	2
Beaker 1 000 ml	2
Beaker 500 ml	5
Beaker 250 ml	10
Beaker 100 ml	50
Beaker 50 ml	10
Beaker 25 ml	10
Beaker 10 ml	10
Beaker 5 ml	10
Cylinder: 10, 25, 50, 100, 250, 500 & 1 000 ml capacity	2 each
Microscope slide:	2 packs of 100 each
- type: plain	
- grade: super premium	
- thickness number: 1	
Cover-glass, square packed in plastic hinged-lid dispenser box:	2 packs of 200 each
- size: 18 × 18 mm	
- thickness number: 1	
Cover-glass, square packed in plastic hinged-lid dispenser box:	2 packs of 200 each
- size: 24 × 24 mm	
- thickness number: 1	
Pipette, graduated type 3 Class B:	1 pack of 5
Calibrated to deliver from zero at the top to any graduation line down to the jet, capacity 5 ml	
Test-tube, heavy-wall, heat-resistant glass without rim:	1 pack of 100
Size: 150x16 mm	
Petri dish, heat-resistant glass, complete with lid:	1 pack of 200
- diameter of bottom dish: 94 mm	
- diameter of lid: 100 mm	
Petri dish, shatterproof, transparent, chemically resistant, useful for biotechnology as resists cell adherence, autoclavable; diameter 100 mm, height 15 mm	20 packs of 10 each

Annex 6
Summary of plant nutrient estimation methods

TABLE A6.1
Plant nutrient estimation methods

Plant nutrients/forms	Soil	Mineral fertilizers	Plants
Total N	Kjeldahl method	Kjeldahl method	Kjeldahl method
Mineralizable N	Alkaline $KMnO_4$ method	-	-
NO_3-N	Phenoldisulphonic acid method	Estimation of NH_4+NO_3-N (below) - estimation of NH_4-N (below)	-
NH_4-N	Indophenol blue method	Distillation with MgO/alkali	-
NH_4+NO_3-N	-	Devarda's treatment followed by distillation with MgO/alkali	-
P	Available P:	Total P:	Total P:
	Bray's method No. 1 for acid soils	Gravimetric quinolinium phosphomolybdate method	Spectrophotometric vanadium phosphomolybdate method
	Olsen's method for alkali soils	Volumetric ammonium phosphomolybdate method	
K	Available K:	Total K:	Total K:
	Flame photometric method (neutral ammonium acetate extraction)	Sodium tetraphenyl boron (STPB) method	Estimation by AAS
S	Available S:	-	Total S:
	Spectrophotometric barium sulphate precipitation method		Spectrophotometric barium sulphate precipitation method
Ca/Mg	EDTA titration method	-	Estimation by AAS
			EDTA titration method
Zn, Cu, Fe & Mn	DTPA extraction and estimation by AAS	Estimation by AAS	Estimation by AAS
B	Hot water extraction and estimation by AAS	Estimation by AAS	Estimation by AAS
	Hot water extraction and determination by azomethine-H colorimetric method		Colorimetric method as in soils
Mo	Ammonium acetate extraction and estimation by AAS	Estimation by AAS	Estimation by AAS Colorimetric method as in soils
	Ammonium oxalate extraction and estimation by colorimetric method		

Annex 7
Automation of analytical procedures

In view of the accepted importance of analysis, the workload on soil, plant and water testing laboratories has increased considerably in recent years. Therefore, analytical methods have needed to be accelerated through the automation of instruments. Almost all instruments either contain inbuilt computers or can interface directly with microprocessors, thereby simplifying instrument operation and providing versatility for the analyst. Most operations can be performed through keyboard commands. This type of instrument automation enables:

- higher working speeds;
- lower labour requirements;
- added consistency and accuracy;
- flexible and extensive data processing;
- various modes of display;
- potential for unattended measurements.

Instrument-based methods that can be automated relatively easily are:

- atomic absorption spectrometry;
- inductively coupled plasma (ICP) emission spectrometry;
- mass spectrometry (MS);
- near infrared reflectance spectrometry (NIR);
- ion chromatography (IC);
- electrochemical methods (to a lesser extent).

In combination with the above techniques, continuous flow analysis (CFA) and flow injection analysis (FIA) are often used. An ICP emission spectrophotometer cannot be operated without the use of microprocessors. The microprocessor (with the help of software) enables the automatic sampling, setting of instrument operating parameters, calibration, result evaluation, data storage and retrieval, data transfer, etc. required for the operation of the instrument for the analysis of a particular element.

The entire laboratory operation from sample preparation to calculating and reporting of concentration of analyte can be automated with the help of robotic systems, which are available and undergoing continuous development. The type of robot that may work best in a soil testing laboratory is one that travels on a track, the length of which can be essentially unlimited (Munter, 1990).

Through the use of a laboratory information management system (LIMS), the automatic control of the functioning of the system, computations and data

management can be carried out efficiently. Skotnikov (1998) developed an automated workstation for soil analysis.

The methods and uses of some modern multi-element analysing are discussed below (adapted from Singh, Chhonkar and Pandey, 1999). Each item of equipment has an operational manual supplied by the manufacturers.

AUTOANALYSERS

Autoanalysers are versatile modularized instruments used for automatic chemical analysis of soil, plant, water and fertilizer samples. These systems are mainly of two types, i.e. CFA and FIA. They are designed to offer automatic simulation of operations used in manual procedures for the estimation of an element on a conveyer-belt principle. Usually, autoanalysers use the same reagents as those used in manual assays.

Principle

In autoanalysers based on the CFA mechanism, the samples are loaded into cups or test-tubes on the sampler. The samples and a number of streams of reagents are made to flow from one module to the next through plastic or glass tubings by the action of multiple-channel peristaltic pumps operating continuously. Each module automatically performs a different function in the analysis. Air bubbles are added to the flowing analytical stream to segment the streams of samples and reagents (Ferreira, Rangel and Lima, 1998). The samples and reagents are brought together under controlled conditions in the mixing coils that are part of the manifold, causing a chemical reaction that produces colour.

The FIA system is similar to the CFA system in its modules. However, there is no air segmentation and the sample is injected rapidly into the carrier stream via an injection valve or syringe as a “plug”.

Operating procedure

The operating procedure is:

1. Prepare working standards of required concentrations, reagents and carrier solutions.
2. Switch on the sampler, analyser and spectrophotometer and allow at least 30 minutes to warm up.
3. Set the wavelength, gain factor, pump times, injection valve cycle, plotting parameter, etc. through keyboard commands of the modules as per the requirement of the analysis. Where the instrument interfaces with a microprocessor, the above parameters may be set automatically by the default choice.
4. Place the blank, working standards and samples in cups or test-tubes on circular tray of sampler in proper sequence.
5. Place the carrier and reagent solution bottle in the holder.
6. Loosen the tube holders of the pumps and insert the pump tubes. Connect the aspiration ends of all the pump tubes except one to a bottle containing

degassed water. Then press the ends of all the pump tubes except one, which is connected to the manifold (chemifold). Connect the aspiration end of the remaining pump tube to the injection valve outlet and its pressed end to the waste tube.

7. Attach the pump tube holders, and release the tension by turning the tension screws counter-clockwise.
8. Lubricate the pump tubes and the rollers with a small amount of silicon oil.
9. Assemble the manifold (chemifold) as per the requirement of the procedure and flow diagram. The reaction coils and tubes used for various connections should be checked carefully according to the prescribed colour code.
10. Connect the inlet stream of the flow cell to the manifold, and the outstream to the waste bottle.
11. Check the flow pattern of the carrier stream by running the pumps. Apply sufficient pressure on the pump tube holders by turning the tension screws clockwise until liquid starts flowing through the pump tubes. In the event of any leakage, stop the pump and correct it.
12. After checking the flow pattern, remove the aspiration end of the pump tube from degassed water bottle and connect it to the carrier and reagent solution bottles according to the requirement of the method (see application note). Now the instrument is ready for the actual run.
13. Start the pumps and set the baseline zero from the keyboard on the spectrophotometer by injecting the blank. Then, run the instrument for calibration and sample analysis.
14. Generate a calibration curve by recording absorbance from the display of the recorder of a series of standard solutions of increasing concentrations. From the absorbance of the test solution, determine the concentration using the calibration curve. With microprocessor-interfaced instruments, the sample peaks are compared automatically with the calibration curve after being corrected for baseline and sensitivity drift, and the results are displayed on the monitor screen.
15. If the instrument shows signs of "over" or "error", then dilute the samples considering the observed absorbance of the sample, and feed the sample again.

Switching off

The procedure for closing down the instrument is:

1. Before closing down, operate the instrument using degassed distilled water from all the channels until the detector reading returns to zero.
2. Loosen the pump tube holders immediately after the analysis.
3. Switch off the instrument in the following order: spectrophotometer, analyser, sampler and computer.

Precautions

It is important to observe the following precautions at all times:

- Never use any component of the instrument if it is in need of adjustment and/or repair.
- Always keep the equipment clean. It is important to clean the sampler and manifold immediately after the analysis is over.
- Clean the rollers and the pump tube holders every month. Lubricate the rollers and the pump tube holders with silicon oil regularly.
- Replace flattened and dirty pump tubes. While using new pump tubes, care should be taken for the change in the flow rate.
- Use all the tubings of prescribed diameter or colour code for a particular method as given in the operating manual.
- Check all the tubings before use for clogging of the passage.
- Develop different manifold (chemifold) units for different analytical methods in order to avoid sample contamination.
- Use solvent-resistant manifold for organic solvents. Never use organic solvent on perspex manifold, not even for cleaning stains.
- Check the manifold bores regularly for plugging. Use a nylon wire or steel to remove solid particles.
- Keep the external walls of the flow cell absolutely clean. Fingerprints, grease, etc. can be removed using tissue paper soaked in acetone or absolute ethanol.
- Care should be taken in handling cadmium during column preparation as it is toxic.

ATOMIC ABSORPTION SPECTROPHOTOMETER

Principle

In an analysis employing an atomic absorption spectrophotometer (AAS), the sample in the form of a homogeneous liquid is aspirated into a flame where “free” atoms of the element to be analysed are created. A light source (hollow cathode lamp) is used to excite the free atoms formed in the flame by the absorption of the electromagnetic radiation. The decrease in energy (absorption) is then measured. It follows the Lambert–Beer law, i.e. the absorbance is proportional to the number of free atoms in the ground state (Baker and Suhr, 1982).

Preparation of standards and sample solutions

The procedure is:

1. Prepare stock standards in concentrations of 1 000 mg/litre from pure metal wire, granules, foil, metal oxides or other suitable primary standard compounds of the elements.
2. Prepare a blank sample using the same reagents as used for standard samples but without the elements intended to be estimated.

3. Free the sample solution from interfering elements and suspended solids, which may cause clogging of the nebulizer.
4. Adjust both standards and unknowns to a concentration range that is compatible with the analytical range of the instrument. It should preferably be at least 5–10 times the detection limit of the instrument.

Instrument operation

The operating procedure is:

1. Check the instrument for the proper fitting of all the tubings, required type of burner (air acetylene or nitrous oxide acetylene) and hollow cathode lamps.
2. Fill the liquid trap with the solvent to be used for the analysis.
3. Align the hollow cathode lamp of the element to be analysed with the optical path of the instrument by rotating the lamp turret.
4. Switch on the instrument and allow at least 30 minutes for warming up.
5. Switch on the deuterium lamp for background correction, which is generally required when the wavelength of the resonance line of the element is less than 250 nm.
6. Use the lamp current recommended by the lamp manufacturer.
7. Select the desirable wavelength and the bandpass width or slit width.
8. Optimize the burner position by using the vertical, horizontal and rotational adjustment knobs until the burner slot is aligned with the beam and is just below the position from where it starts to block the light path.
9. Switch on the compressor in order to generate air supply in the case of an air acetylene flame. If an N_2O acetylene flame is used, turn on the N_2O supply cylinder. Select air with the support selector knob. Adjust the support flow (air) reading to between 6 and 9 flow units.
10. If a nitrous oxide acetylene flame is used, then first ignite an air acetylene flame and then change over to a nitrous oxide acetylene flame.
11. Turn on the gas supply from cylinder, use the fuel-control knob of the instrument and light the flame.
12. Adjust the fuel control (acetylene) and support control (air or nitrous oxide) knobs to produce the required kind of flame of air acetylene or nitrous oxide acetylene flame.
13. Set the instrument to zero by means of the "zero" control against a reagent "blank" solution.
14. Aspirate a standard (or sample) and optimize fuel, oxidant and sample flow rates by adjusting the fuel knob, fuel support knob and nebulizer so that a maximum signal (absorbance) is achieved.
15. Prepare a calibration curve by recording the absorbance of a series of working standards. The calibration must be done for each set of analysis.
16. If the instrument shows signs of "over" or "error", then dilute the samples depending on the absorbance of the sample, and feed the sample again.

17. If the instrument has been used in a higher concentration range, then operate the instrument using distilled water until the reading returns to zero, before closing down.

Switching off

The procedure for closing down the instrument is:

1. Turn off the gas from the cylinder.
2. Wait for extinction of the flame, and then turn off the fuel-control knob.
3. Turn off the air compressor and fuel-support knob.
4. The shutdown sequence for a nitrous oxide acetylene flame involves first changing over to an air acetylene flame and then extinguishing it.
5. Switch off the instrument.

Precautions

It is important to observe the following precautions at all times:

- Acetylene cylinders should always be used in a vertical position in order to prevent liquid acetone entering the gas line.
- Acetylene cylinders should not be run at a pressure lower than 500 kPa. Never operate acetylene lines at more than 100 kPa. At higher pressures, acetylene can decompose or explode spontaneously.
- Never run the nitrous oxide acetylene flame without the "red feather" visible, or with less than five flow units of acetylene.
- Do not leave uncovered containers of the volatile organic solvents near the uncovered flame.
- Do not look at flame without the aid of safety glasses or the flame shield.
- Do not leave the flame completely unattended.
- Do not ignite the flame if the air flow is below six flow units.
- Do not adjust the air (or N_2O) and gas supply to alter the sensitivity of the instrument after calibration of the instrument.

INDUCTIVELY COUPLED PLASMA – ATOMIC EMISSION SPECTROSCOPY

A new analytical technique called inductively coupled plasma – atomic emission spectroscopy (ICP–AES) is used for simultaneous multielement analysis of biological materials and soils. This technique offers advantages over an AAS and other multielement methods because matrix problems are eliminated or minimized through the use of the high-temperature argon plasma. Apart from multielement capability at all concentration levels, plasmas are noted for their relative freedom from the chemical and ionization interferences that are common with an AAS. The detection limits are equal to or better than an AAS (depending on the element analysed). Elements such as aluminium, phosphorus, sulphur and boron, which are either poorly measured at low concentrations or not possible with an AAS, are readily determined with higher sensitivity by ICP–AES.

Principle

The ICP–AES method is based on the observation of atomic emission spectra when samples in the form of an aerosol, thermally generated vapour or powder are injected into an ICP atomization and excitation source. By definition, plasma refers to a hot gas in which a significant fraction of the atoms or molecules is ionized. Plasmas are electrically conducted and have been referred to as electrical flames, as no combustion takes place. This is because instruments using a plasma source generally use inert argon gas.

The ICP is produced by passing initially ionized argon gas through a quartz torch located within an induction coil (copper coil) that is connected to a radio frequency (RF) generator. The RF generator produces 1.5–3 kW of power at a frequency of 27.1 MHz. An oscillating magnetic field is formed within the quartz torch in response to the RF energy passing through the coil. Electrons and ions passing through the oscillating electromagnetic field flow at high acceleration rates within the quartz torch space. As argon gas enters the magnetic field associated with the induction coil, its atoms collide with the accelerated ions and electrons, resulting in the ionization of the argon gas. These collisions give rise to ohmic heating, which produces plasma with temperatures ranging from 4 000 to 6 000 °C. The resultant plasma is contained within the torch by means of argon flow.

The method of presenting the sample to the plasma is similar to that used in flame atomic absorption. The liquid sample is aspirated into the plasma through a nebulizer system, using argon carrier gas at a rate of about 1 litre of argon per minute. The prevailing high temperature in the plasma leads to complete vaporization, atomization and excitation of the element to be analysed. The exited neutral atoms or ions of the sample emit radiation of characteristic wavelengths. The intensity of the emitted radiation is measured by the spectrophotometer component of the ICP–AES instrument.

Preparation of soil and plant samples

The digestion of soil and plant samples for total elemental analysis by ICP–AES is similar to that used for other emission instruments. Universal/multielement soil extractants are used for the extraction of soil samples. Recently, acidified AB-DTPA and Mehlich No. 1 extracts have been analysed by ICP–AES (Soltanpour *et al.*, 1998).

Preparation of standard solution

The procedure for the preparation of stock standard solution containing 1 000 mg/litre of an element from pure metal wire or suitable compounds of the element is similar to that described for AAS. However, multielement working standard solutions (secondary standards) should be made in such a way that these contain the maximum number of the elements compatible with stability considerations and match with the sample solutions in kind and strength of acids. In soil and plant analysis, one set of secondary standards is required for each multielement extracting solution, and one for each soil and plant digest. McQuaker, Kluckner

and Chang (1979) devised a calibration scheme for 30 elements that satisfies the needs of analysts engaged in soil, water, tissue, and particulate matter analysis.

Instrument operation

The general principles that allow the determination of the optimal analytical conditions for the operation of ICP–AES are similar to those for AAS. Microprocessors are used for the automatic control of instrument components, setup and optimization of required operational parameters, instrument calibration, and manipulation and storage of data through key commands. As the actual operation of different ICP spectrometers varies with the type, make and computer software of the instrument, the operation manual provided by the manufacturers should be consulted. Usually, when an ICP spectrophotometer is purchased, the manufacturer/dealer provides training to the analysts on operating procedures.

Precautions

It is important to observe the following precautions at all times:

- Filter soil extracts and soil and plant tissue digests with No. 42 filter paper to prevent clogging of the nebulizer.
- To prevent clogging of the nebulizer tip, use either a high salt nebulizer (Babington-type) or standards and samples with a very low salt content.
- Avoid mixing chemicals that cause precipitation during the preparation of multielement working standard solutions.

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Annex 8
Examples of laboratory registers

TABLE A8.1
Equipment register

Item	Description	Qty	Reference	Model	Price/unit *(US$)*	Total price *(US$)*
1	Spectrophotometer	1	BDH/331/1042/04	1112	3 620	3 620
2	Flame photometer					
3						
4						
5						

TABLE A8.2
Glassware register

Item	Description	Qty	Reference	Model	Price/unit *(US$)*	Total price *(US$)*
1	Beaker 5 ml	30	BDH/209/0310/01	Abc	5	150
2	Flask					
3						
4						
5						

TABLE A8.3
Chemicals register

Item	Description	Qty	Reference	Model	Price/unit *(US$)*	Total price *(US$)*
1	Hydrochloric acid	2 litres	BDH-10125-5Y	AR	16	32
2	Sulphuric acid					
3						
4						
5						

TABLE A8.4
Register for miscellaneous items

Item	Description	Qty	Reference	Model	Price/unit *(US$)*	Total price *(US$)*
1	Funnel stand	2	ABC	Wooden	5	10
2						
3						
4						
5						

Annex 9

Grades of chemicals and glassware

TABLE A9.1
Grades of chemicals

Grade	Purity	Notes
Technical or commercial	Indeterminate quality	For use in preparation of cleaning solution only.
Chemically pure (CP)/ Laboratory reagent (LR)	More refined, but still unknown quality	For general chemical use where very high purity is not required. It is also used in training and for laboratory practice.
Specially pure (SP)	Meets minimum purity standards	Conforms to tolerance set by the United States Pharmacopeia for contaminants dangerous to health.
Analytical reagent (AR)	High purity	Conforms to minimum specifications set by the Committee on Analytical Reagents of the American Chemical Society.
Primary standard	Highest purity	Required for accurate volumetric analysis (for standard solutions). Composition of primary standards does not undergo change. Sodium carbonate and borax are primary standards of bases. Potassium hydrogen phthalate is used as primary standard acid.

TABLE A9.2
Tolerances for volumetric glassware, Class A

Capacity		**Tolerance**	
(less than and Including)	Volumetric flask	Transfer pipette	Burette
(ml)		*(ml)*	
1 000	±0.30	-	-
500	±0.15	-	-
100	±0.08	±0.08	±0.10
50	±0.05	±0.05	±0.05
25	±0.03	±0.03	±0.03
10	±0.02	±0.02	±0.02
5	±0.02	±0.01	±0.01
2	-	±0.006	-

Annex 10
Equivalent and molecular weights of compounds

TABLE A10.1
Equivalent and molecular weights of some important compounds needed in chemical analysis

Compound	Formula	Molecular weight	Equivalent weight
		(g)	*(g)*
Ammonium acetate	CH_3COONH_4	77.08	77.08
Ammonium chloride	NH_4Cl	53.49	53.49
Ammonium fluoride	NH_4F	37.04	37.04
Ammonium nitrate	NH_4NO_3	80.04	80.04
Barium chloride	$BaCl_2.2H_2O$	244.28	122.14
Boric acid	H_3BO_3	61.83	20.61
Calcium acetate	$(CH_3COO)_2Ca$	158.00	79.00
Calcium carbonate	$CaCO_3$	100.09	50.05
Calcium chloride (dihydrate)	$CaCl_2.2H_2O$	147.02	73.51
Calcium hydroxide	$Ca(OH)_2$	74.00	37.00
Calcium nitrate	$Ca(NO_3)_2$	164.00	82.00
Calcium sulphate	$CaSO_4.2H_2O$	172.17	86.08
Ferrous ammonium sulphate	$FeSO_4.(NH_4)_2SO_4.H_2O$	392.13	392.13
Ferrous sulphate	$FeSO_4.7H_2O$	278.01	139.00
Magnesium chloride	$MgCl_2.6H_2O$	203.30	101.65
Magnesium nitrate	$Mg(NO_3)_2.6H_2O$	256.41	128.20
Potassium chloride	KCl	74.55	74.55
Potassium dichromate	$K_2Cr_2O_7$	294.19	49.04
Potassium hydroxide	KOH	56.10	56.10
Potassium permanganate	$KMnO_4$	158.03	31.60
Potassium nitrate	KNO_3	101.10	101.10
Potassium sulphate	K_2SO_4	174.27	87.13
Potassium hydrogen phthalate	$COOH\ C_6H_4\ COOK$	204.22	204.22
Oxalic acid	$C_2H_2O_4.2H_2O$	126.00	63.00
Silver nitrate	$AgNO_3$	169.87	169.87
Sodium acetate (anhydrous)	CH_3COONa	82.04	82.04
Sodium bicarbonate	$NaHCO_3$	84.01	84.01
Sodium carbonate	Na_2CO_3	106.00	53.00
Sodium chloride	$NaCl$	58.45	58.45
Sodium hydroxide	$NaOH$	40.00	40.00
Sodium nitrate	$NaNO_3$	84.99	84.99
Sodium oxalate	$Na_2C_2O_4$	134.00	67.00
Sodium sulphate	Na_2SO_4	142.04	71.02
Sodium thiosulphate	$Na_2S_2O_3.5H_2O$	248.18	248.18

Annex 11
Soil sample information sheet

FIGURE A11.1
Soil sample information sheet

Sample No. ____________
Name of sample collector ____
Address ____ Date ________
Area __________________________ Location _________________________
Name of farmer ______________________ Farm size ____________________
Vegetative cover ______________________
Source of water ___________________ Water quality _____________________
Sample depth ____________________ Previous crop _____________________

Purpose of analysis:	Land capability assessment	Slope:	1–2 percent
	Fertility evaluation and fert. recommendation		2-5 percent
			5-10 percent
	Salinity appraisal and causes of the source of salinity, if known		10-25 percent
			> 25 percent
	Soil classification		

Irrigation method:	Flood	Years of irrigation:	Never irrigated
	Furrow		1–5
	Sprinkler		5–15
	Drip		
	Rainfed		

Years of cultivation:	Never cultivated	Drainage:	Good
	1–5		Moderate
	5–15		Poor
	>15		

Manure used in the previous crop and dose __________________________

Fertilizers used in the previous crop and dose _________________________

Annex 12
Colour change of solutions owing to pH change

TABLE A12.1
Colour changes owing to pH changes in the presence of pH indicators

pH indicator		pH transition intervals		
Name	**Colour**	**pH**	**pH**	**Colour**
Cersol red	Pink	0.2	1.8	Yellow
m-Cresol purple	Red	1.2	2.8	Yellow
Thymol blue	Red	1.2	2.8	Yellow
2,4-Dinitrophenol	Colourless	2.8	4.7	Yellow
Bromochlorophenol blue	Yellow	3.0	4.6	Purple
Bromophenol blue	Yellow	3.0	4.6	Purple
Methyl orange	Red	3.1	4.4	Yellow-orange
Bromocresol green	Yellow	3.8	5.4	Blue
2,5-Dinitrophenol	Colourless	4.0	5.8	Yellow
Methyl red	Red	4.4	6.2	Yellow-orange
Chlorophenol red	Yellow	4.8	6.4	Purple
Litmus extra pure	Red	5.0	8.0	Blue
Bromophenol red	Orange-yellow	5.2	6.8	Purple
Bromocresol purple	Yellow	5.2	6.8	Purple
4-Nitrophenol	Colourless	5.4	7.5	Yellow
Bromoxylenol blue	Yellow	5.7	7.4	Blue
Bromothymol blue	Yellow	6.0	7.6	Blue
Phenol red	Yellow	6.4	8.2	Red
3-Nitrophenol	Colourless	6.6	8.6	Yellow-orange
Cresol red	Orange	7.0	8.8	Purple
1-Naphtholphthalein	Brownish	7.1	8.3	Blue-green
Thymol blue	Yellow	8.0	9.6	Blue
Phenolphthalein	Colourless	8.2	9.8	Red-violet
Thymolphthalein	Colourless	9.3	10.5	Blue

Annex 13
Glossary of biofertilizer terms

Actinomycetes: A member of the bacterial order Actinomycetales, representing transition between bacteria and fungi, characterized by radiating arrangement of the mycelium and by small spores. They differ from fungi in that they are prokaryotic, have bacterial-type cell walls and are inhibited by antibacterial agents. Most actinomycetes are saprophytic and aerobic. Some (e.g. *Frankia*) are known to fix N in association with higher plants.
Activated charcoal: Charcoal that has been treated to remove impurities and hydrocarbons in order to increase its purity and adsorptive capacity. Inhibitory substances in the nutrient medium may be adsorbed onto charcoal included in the medium. It acts by condensing and holding a gas or solute on its surface. It may be of different origin and variable composition.
Aerobe: An organism that grows best in the presence of free oxygen, e.g. *Rhizobium* and *Azotobacter*.
Agar or agar-agar: A gelatinous polysaccharide obtained from Ceylon moss (red algae, Rhodophyceae). It is a solidifying agent and, thus, when mixed with nutrient media (at 0.6–1.5 percent), it forms a gel. Its firmness is affected by pH and salt concentration of the medium. It is softer when the medium is more dilute and acidic. Agar gel solidifies at about 38 °C and melts at about 100 °C. It is a base for several solid and semi-solid media.
Agar plate count: The number of bacterial colonies that develop on an agar-containing medium in a Petri dish seeded with a known amount of inoculum. From the count, the concentration of bacteria per unit volume of inoculum can be determined.
Anaerobe: An organism that lives and grows in the absence of free oxygen. Anaerobes are either obligatory anaerobes, which grow only in the absence of oxygen, e.g. *Clostridium tetani*, or facultative anaerobes, which can grow either in the presence or absence of oxygen, e.g. *Escherichia coli*.
Anaerobic microbes: Microbes capable of obtaining oxygen for their growth by reducing oxygen-containing compounds in the soil. They are not dependent on a supply of free oxygen.
Autoclave: An enclosed chamber for heating substances above their boiling points under pressure using steam to sterilize liquids, glassware, etc.
Azotobacter: Free-living (non-symbiotic) N-fixing aerobic bacteria. They are plemorphic with ovoid cells, and are relatively large organisms measuring 2.0–7.0 × 1.0–2.5 µm. Cell size and shape vary considerably with species, strain, age of culture, and growth conditions. The seven species of *Azotobacter* on the basis of cell shape, pigmentation and mobility are *A. chroococcum*, *A. beijerinckii*, *A. agilis*,

A. macrocytogenes, *A. paspali, A. insignis* and *A. vinelandi*. Among the important species, *A. beijerinckii* is non-mobile and ovoid rod-shaped. Cells occur singly, in pairs and sometimes in chains with large capsules. As cultures age, the cells become coccoid, form cysts and turn yellow or cinnamon with a water-insoluble pigment.

Bacterial count: Number of bacteria per unit of volume of a substance.

Bacterial culture: Any medium enriched with any particular bacteria.

Bacterial population: A group of bacteria belonging to the same species and exchanging genetic material among one another but with little contact with other groups of the same species. The population is expressed as 10^4/g, 10^5/g, etc.

Batch fermentation: A fermentation system run as a batch culture in which the reactor is charged with the substrate, and microbial inoculum is added with the substrate. The fermentation process is allowed to continue for 4–10 days until completed. After completion of fermentation, the cells are removed from the broth medium.

Biological N_2 fixation (BNF): N fixation by biological process as distinguished from chemical N fixation in a fertilizer factory. It is mediated by several micro-organisms. *Rhizobium*, blue-green algae (BGA), *Azotobacter* and *Azospirillum* are important in BNF. In *Rhizobium*–legume symbiosis, atmospheric N makes its way through the soil to the nodules, where it is reduced to ammonia by the nitrogenase of the *Rhizobium* bacteroids. This ammonia is subsequently incorporated into carbon skeletons to produce amino acids and proteins.

Broth: A suspension of micro-organism culture. Broths are very common in the laboratory e.g. *Rhizobium* broth.

Carbon source: A source that provides C to organisms for their growth. For photosynthetic and autotrophic organisms, the C source is generally carbon dioxide. For many heterotrophic organisms, it is usually glucose or sucrose.

Carrier: Substances used to contain and carry the cultures of micro-organisms in a commercial biofertilizer product, e.g. peat, lignite, charcoal, rice husk, vermiculite, soil, coir dust, press mud, and polymer compounds. A good carrier should be: (i) chemically inert; (ii) able to support growth of organisms in storage; (iii) have a high moisture holding capacity; and (iv) have no toxic influence on the organisms it carries. Most biofertilizers except liquid biofertilizer are carrier-based. Carriers giving an acid reaction (e.g. peat and lignite), depending on the acidity, are mixed with powdered calcium carbonate to be neutralized. Carriers may be sterile or unsterile. Sterilized carriers are desirable because unsterile carriers contain more contaminants. Sterile carriers have been either irradiated or heat-sterilized. After growing the microbial broth in fermenters, the micro-organisms are mixed with the carrier. Good carriers can support 1 000 million (10^9) cells/g when the inoculant is fresh and maintain 100 million (10^8) cells/g for six months after production. Before use, the carrier should be dried and ground. After mixing, carriers should be allowed time for curing in order to dissipate the heat generated during mixing.

Cell count: The number of cells per unit of suspension. The cell numbers are estimated with a haemocytometer.
Cell counter: An automated device to count the number of cells in a sample. The simpler cell counters consist of a mechanism to draw a known volume of a suitably diluted cell culture through a detection device.
Charcoal: A variety of carbon. It is black, porous, imperfectly combusted OM, similar to burned wood. It has adsorbent and filtering qualities. It is used as a carrier for biofertilizers.
Colony: A contiguous group of single cells derived from a single ancestor and growing on a solid surface.
Congo red test: This test differentiates Rhizobia from other contaminants. An aliquot of 25 ml of 1-percent solution of Congo red (a basic dye) in water is added to 1 litre of yeast extract mannitol agar (YEMA). On this medium, when suspected strains of nodule bacteria are plated, Rhizobia stand out as white, translucent, glistening, elevated and comparatively small colonies with entire margins in contrast to strained colonies of other contaminants.
Contaminant: An undesirable bacterial, fungal or algal micro-organism accidentally introduced into a culture or culture medium. It may over grow the cells of interest or inhibit their growth by releasing toxic metabolites. In biofertilizer production, no contaminants are desirable.
Contamination: Accidental introduction of an undesirable substance or organism into a medium of culture.
Continuous fermentation: Process in which the cells are kept in a state of exponential growth, or in which stationary cells produce a secondary product continuously. In general, a suitable medium is fed into the fermenter at the same rate as the effluent is removed so that conditions remain constant.
Culture: A term referring to the growth of micro-organisms under artificial conditions, or the results of microbial growth on artificial culture media.
Culture medium: Any material in which micro-organisms find nourishment and in which they can reproduce (plural is media).
Culture room: A controlled-environment room (light, temperature and relative humidity) for incubation of cultures.
Dry cell weight: The weight of cells contained in an aliquot obtained after drying to constant weight in an oven at 105 °C. Dry weights are often expressed in terms of grams of material per litre or cubic metre of fermenter volume.
In vitro: Refers to biological experiments performed in test-tubes or other laboratory glassware.
In vivo: Refers to laboratory testing of agents within a living organism.
Incubation: Holding cultures of micro-organisms under conditions favourable for their growth. Specialized incubation permits control of humidity, light and temperature. Incubation is usually achieved within closed, head-insulated, thermostatically-controlled chambers.
Inoculant: Culture of microbes (*Rhizobium*, *Azotobacter*, BGA, phosphate-solubilizing microbes, etc.) used for inoculation at field level. Biofertilizers are

inoculants. Inoculants are made available to end users in many forms. They include agar-slope-based cultures, liquid-paraffin-covered agar cultures, lyophilized cultures, carrier-based cultures (peat, charcoal. etc.), and liquid cultures.

Inoculant quality: Describes the population and effectiveness of micro-organisms in an inoculant. A good-quality inoculant contains 10^8 viable cells per gram.

Inoculation: Artificial introduction of micro-organisms onto or into a medium. Inoculation is carried out using an aseptic technique.

Isolate: To set apart, place or separate from others and make a pure culture. The process is isolation.

Isolation medium: A medium suitable for the survival and development of micro-organisms.

Gram negative: Bacteria that lose the colour of the initial Gram stain (e.g. crystal violet) and take the colour of the final stain (safranin). Example: *Rhizobium*.

Gram positive: Bacteria that take the colour of the initial Gram stain and are not decolorized, and do not take the colour of the final stain, i.e. counter-stain. Example: *Bacillus*.

Growth: An irreversible increase in cell size and/or number resulting from cell division or enlargement, usually accompanied by differentiation and an increase in mass.

HEPA filter: An acronym for high-efficiency particulate air filter. A filter capable of screening out particles larger than 0.3 µm. HEPA filters are used in laminar air-flow cabinets (hoods) for sterile transfer work.

Hoffer's alkaline broth test: Test based on the fact that agrobacteria grow at higher pH levels (pH 11), while Rhizobia are unable to do so. The media with pH 11 is used to screen new isolates of nodule bacteria for the purpose.

Liquid medium: Medium not solidified with gelling agent such as agar.

Microbiological tests: Methods of examining specimen objects or materials to determine the presence or absence of micro-organisms, their taxonomic identification, and/or their relative frequency, types and activity.

Mixed culture: Mixture of two or more species of micro-organisms growing together.

Mother culture: Inoculant production starts with a pure slant culture containing the original strain. It may also be termed starter culture.

Plating: A technique used to obtain pure cultures of micro-organisms (bacteria, yeast and fungi) that produce a distinct colony when grown on a solid medium.

Population: Total number of organisms of one kind. The infinite group from which a sample might be taken.

Shelf-life: The period up to which a product (biofertilizer) contains a certain minimum specified number of viable organisms. Products are not useful beyond their shelf-life.

Staining: A method of preparing samples that enhances the contrast when examined under a microscope.

Starter culture: Pure culture or mixture of micro-organisms used for starting a fermentation process. It may also be termed mother culture.

Sterile: Free from infectious matter, agents or living micro-organisms.
Sterilization: The process of making materials sterile through killing or excluding micro-organisms or their spores with heat, filters, chemicals or other sterilants.
Stock culture: Known species of micro-organisms maintained in the laboratory for various tests and studies.
Strain: Pure culture of an organism composed of the descendants of a single isolation. The term also refers to a cell or population of cells that exhibit a particular named characteristic.
Substrate: The medium on which a fungus and bacteria grow, especially in culture. The substance or the object on which an organism lives and from which it obtains its nourishment.
Subculture: A culture derived from another culture or the aseptic division and transfer of a culture or a portion of that culture (inoculum) to a fresh nutrient medium. Subculturing is usually done at set time intervals, the length of which is called the subculture interval or passage time.
Viable count: A determination of the number of cells in a population that is capable of growth and reproduction.
Yield (bacterial): The yield is the difference between the initial and the maximum bacterial mass. It is expressed in grams of dry weight. The yield is also frequently related to the concentration of the substrate and calculated as the molar growth yield (grams of cells per mole of substrate).

Annex 14
Units and conversion factors

TABLE A14.1
Units and conversion factors

To convert unit in Column 1 to unit in Column 2 multiply by:	Column 1: SI unit	Column 2: non-SI unit	To convert unit in Column 2 to unit in Column 1 multiply by:
		Area	
2.47	hectare, ha	acre	0.405
247	square kilometre, km^2 $(10^3\ m)^2$	acre	$4.05x\ 10^{-3}$
0.386	square kilometre, km^2 $(10^3\ m)^2$	square mile, mi^2	2.590
$2.47x\ 10^{-4}$	square metre, m^2	acre	$4.05x10^3$
10.76	square metre, m^2	square foot, ft^2	$9.29x\ 10^{-2}$
$1.55x\ 10^{-3}$	square millimetre, mm^2 $(10^{-3}\ m)^2$	square inch, in^2	645
		Volume	
$9.73x\ 10^{-3}$	cubic metre, m^3	acre-inch	102.8
35.3	cubic metre, m^3	cubic foot, ft^3	$2.83x\ 10^{-2}$
$6.10x\ 10^4$	cubic metre, m^3	cubic inch, in^3	$1.64x\ 10^{-5}$
1.057	litre, L $(10^{-3}\ m^3)$	quart (liquid), qt	0.946
$3.53x\ 10^{-2}$	litre, L $(10^{-3}\ m^3)$	cubic foot, ft^3	28.3
0.265	litre, L $(10^{-3}\ m^3)$	gallon	3.78
33.78	litre, L $(10^{-3}\ m^3)$	pint (fluid), pt	0.473
		Mass	
$2.20x\ 10^{-3}$	gram, g $(10^{-3}\ kg)$	pound, lb	454
$3.52x\ 10^{-2}$	gram, g $(10^{-3}\ kg)$	ounce (avdp), oz	28.4
2.205	kilogram, kg	pound, lb	0.454
0.01	kilogram, kg	quintal (metric), q	100
$1.10x\ 10^{-3}$	kilogram, kg	ton (2 000 lb), ton	907
1.102	megagram, Mg (tonne)	ton (U.S.), ton	0.907
1.102	tonne, t	ton (U.S.), ton	0.907
		Length	
0.621	kilometre, km $(10^3\ m)$	mile	1.609
1.094	metre, m	yard, yd	0.914
3.28	metre, m	foot, ft	0.304
$3.94x\ 10^{-2}$	millimetre, mm $(10^{-3}\ m)$	inch, in	25.4
10	nanometre, nm $(19^{-9}\ m)$	angstrom, å	0.1
		Yield and rate	
0.893	kilogram per hectare, kg ha^{-1}	pound per acre, lb $acre^{-1}$	1.12
$7.77x\ 10^{-2}$	kilogram per cubic metre, kg m^{-3}	pound per bushel, lb bu^{-1}	12.87

To convert unit in Column 1 to unit in Column 2 multiply by:	Column 1: SI unit	Column 2: non-SI unit	To convert unit in Column 2 to unit in Column 1 multiply by:
1.49×10^{-2}	kilogram per hectare, kg ha^{-1}	bushel per acre, 60 lb	67.19
1.59×10^{-2}	kilogram per hectare, kg ha^{-1}	bushel per acre, 56 lb	62.71
1.86×10^{-2}	kilogram per hectare, kg ha^{-1}	bushel per acre, 48 lb	53.75
0.107	litre per hectare, L ha^{-1}	gallon per acre	9.35
893	tonne per hectare, t ha^{-1}	pound per acre, lb $acre^{-1}$	1.12×10^{-3}
893	megagram per hectare, Mg ha^{-1}	pound per acre, lb $acre^{-1}$	1.12×10^{-3}
0.446	megagram per hectare, Mg ha^{-1}	ton (2 000 lb) per acre, ton $acre^{-1}$	2.24
2.24	metre per second, m s^{-1}	mile per hour	0.447
	Pressure		
9.90	megaPascal, MPa (10^6 Pa)	atmosphere	0.101
10	megaPascal, MPa (10^6 Pa)	bar	0.1
1.00	megagram per cubic metre, Mg m^{-3}	gram per cubic centimetre, g cm^{-3}	1.00
2.09×10^{-2}	Pascal, Pa	pound per square foot, lb ft^{-2}	47.9
1.45×10^{-4}	Pascal, Pa	pound per square inch, lb in^{-2}	6.90×10^3
	Temperature		
1.00 (K - 273)	Kelvin, K	Celsius, °C	1.00 (°C + 273)
(9/5 °C) + 32	Celsius, °C	Fahrenheit, °F	5/9 (°F - 32)
	Water measurement		
9.73×10^{-3}	cubic metre, m^3	acre-inch, acre-in	102.8
9.81×10^{-3}	cubic metre per hour, m^3 h^{-1}	cubic foot per second, ft^3 s^{-1}	101.9
4.40	cubic metre per hour, m^3 h^{-1}	U.S. gallons per minute, gal min^{-1}	0.227
8.11	hectare-metre, ha-m	acre-foot, acre-ft	0.123
97.28	hectare-metre, ha-m	acre-inch, acre-in	1.03×10^{-2}
8.1×10^{-2}	hectare-centimetre, ha-cm	acre-foot, acre-ft	12.33
	Concentration		
1	centimole per kilogram, cmol kg^{-1} (ion exchange capacity)	milli-equivalents per 100 grams, me 100 g^{-1}	1
0.1	gram per kilogram, g kg^{-1}	percent, %	10
1	milligram per kilogram, mg kg^{-1}	parts per million, ppm	1
	Plant nutrient conversion		
	Element	Oxide	
2.29	P	P_2O_5	0.437
1.20	K	K_2O	0.830
1.39	Ca	CaO	0.715
1.66	Mg	MgO	0.602
1.216	N	NH_3	0.777
4.426	N	NO_3	0.226
6.25	N	Protein	0.160
3.00	S	SO_4	0.330
2.5	S	SO_3	0.440
1.724	Organic C	Organic matter	0.580

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Guide to laboratory establishment for plant nutrient analysis

Integrated nutrient management is a well-accepted approach for the sustainable management of soil productivity and increased crop production. It utilizes well-equipped testing laboratories. These need information on a widely acceptable methodology that can ensure reasonable accuracy, speed and reproducibility of results. The method has to be readily comprehensible to those who need to apply it in a routine manner.
This publication provides practical guidelines on establishing composite service laboratories; information on the basics of an analytical laboratory; simple methods for estimating soil and plant constituents for assessing soil fertility and making nutrient recommendations; standard methods for estimating the parameters and constituents of irrigation water for assessing the quality; methods for analysing mineral fertilizers to judge their quality; methods for the isolation, identification, multiplication and commercial production of agriculturally useful microbial inoculants; and details of the equipment, chemicals and glassware required for a given analytical capacity.

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