COURSE GUIDE

AGR 503
ANALYTICAL TECHNIQUES IN AGRICULTURE

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INTRODUCTION

All scientists of different fields generally depend on analysis as a major means of solving scientific problems. This is to enable them proffer solutions to numerous questions on the content of the materials with which they work. The methods by which these analyses are carried out are referred to as analytical techniques. The types of analytical techniques may vary from field to field. This variation stems primarily from differences in the nature of materials with which people in each field works. Agricultural sciences being a key branch of applied sciences that makes use of the principles of physics, chemistry, biology and mathematics in the study of crops, soil and animal feed and parameters. This is because while the soil consists of matter (living and non-living) interacting together physically, chemically and biologically to provide ecosystem services at all levels of consideration, crops and animals are all made up of living cells and serve as key sources of food and survival to man. For an agricultural scientist to be able therefore to answer questions on the constituents of soil, compositions of crops and animals as well as animal feeds, he needs to carry out one form of analysis or the other. Methods and techniques of analysis in soil, crops and animal science are of very vital importance to students of agricultural sciences.

Prerequisites

The background knowledge from biology, chemistry, biochemistry and geology is required.

WHAT YOU WILL LEARN IN THIS COURSE

The course consists of modules in units and a course guide. This course guide tells you briefly what the course is about, what course materials you will be using and how you can work with these materials. In addition, it advocates some general guidelines for the amount of time you are likely to spend on each unit of the course in order to complete it successfully. It gives you guidance in respect of your Tutor-Marked Assignment in the assignment file. There will be regular tutorial classes that are related to the course. It is advisable for you to attend these tutorial sessions. The course will prepare you for the challenges you will meet in the field of soil pedology and classification.

COURSE AIMS

The aim of the course is not complex. The course aims to provide you with an understanding of soil Survey and Land Evaluation; it also aims to provide you with solutions to problems with soil classification in the field.

COURSE OBJECTIVES

To achieve the aims set out, the course has a set of objectives. Each unit has specific objectives which are included at the beginning of the unit. You should read these objectives before you study the unit. You may wish to refer to them during your study to check on your progress. You should always look at the unit objectives after completion of each unit. By doing so, you would have followed the instructions in the unit. Below are the comprehensive objectives of the course as a whole. By meeting these objectives, you should have achieved the aims of the course as a whole. In addition to the aims above, this course sets to achieve some objectives.

Thus, after going through the course, you should be able to:

- Know the basic principles governing common laboratory equipments
- Know the basic principles governing basic analytical procedures
- Explain precision, accuracy and operational variation in analytical techniques.
- Understand the use of instruments, equipments and machines
- Know how to carry out animal, plant and soil sampling and sample preparation
- Have good idea of major analytical instruments and their principles of operations. Such instruments include; Microscopy, Spectrophotometer, Polymerase chain reaction (PCR), Gel Electrophoresis, High performance liquid Chromatography (HPLC)
- Understand the use of thin layer chromatography (TLC) and Gas Chromatography (GC)
- Explain hematology and serum biochemistry
- Understand the concept of cryopreservation

WORKING THROUGH THE COURSE

To complete this course, you are required to read each study units, read the textbook and other materials which may be provided by the National Open University of Nigeria. Each unit contains self-assessment exercises

and at certain points in the course you would be required to submit assignment for assessment purpose. At the end of the course there is a final examination. The course should take you a total of 17 weeks to complete. Below you will find listed all the components of the course, what you have to do and how should allocate your time to each unit in order to complete the course on time and successfully the details that you spend a lot of time to read. I would advise that you avail yourself the opportunity of attending the tutorial sessions where you have the opportunity of comparing your knowledge with that of other people.

THE COURSE MATERIALS

The main components of the course are:

1. The Course Guide
2. Study Units
3. References/Further Reading
4. Assignments
5. Presentation Schedule

STUDY UNITS

The study units in this course are as follows:

Module 1 Principles Governing Basic Analytical Procedures

Unit 1 Principle of Safety
Unit 2 Principle of Quality
Unit 3 Principle of Precision and Accuracy
Unit 4 Calibration and Use of Blanks in Analytical Procedures

Module 2 Animal, Crop and Soil Samples Analysis

Unit 1 Animal Feed Sample Analysis
Unit 2 Plant Sampling and Sample Preparation for Analysis
Unit 3 Soil Sampling and Sample Preparation

Module 3 Use of Instruments, Equipments and Machines

Unit 1 Microscopy
Unit 2 Principles and Operation of Atomic Absorption Spectrophotometer (AAS)
Unit 3 High-performance liquid chromatography (HPLC)
Unit 4 Thin Layer Chromatography (TLC)
Unit 5 Gas Chromatography
Unit 6 Polymerase Chain Reaction (PCR)

MAIN COURSE

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MODULE 1 PRINCIPLES GOVERNING BASICANALYTICAL PROCEDURES

UNIT 1 PRINCIPLE OF SAFETY

CONTENTS

1.0 INTRODUCTIONS

Safety principles are important consideration in any soil/plant laboratories even though it is frequently overlocked. As the saying goes “Safety first” anyone carrying out any activity in the lab should be very safety conscious. To avoid injuries that may lead to deformation or even death, all safety precaution in the Laboratory must be strictly adhered to. Therefore, safety is of interest to every employee who works in the labs as well as any organisations which operate laboratories. All staff, not minding his/her grade, technical skills or employment status should be properly educated on the subject of safety when laboratories are concerned especially at commencement of work. From time to time, reminders of such regulations should be done to encourage staff familiarity with such regulations.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- understand the safety precautions of a soil science laboratory

- adhere strictly to all safety precautions in the laboratory to avoid any casualty.

3.0 MAIN CONTENT

3.1 Important Facts to Know

- Always double-check the name of the reagent to be used and the name of the reagent you are using. Reagent bottles should remain stoppered, except when you are actually pouring solutions out of them. Always replace the stopper or lid of stock solutions or stains. Be sure to put them on the container they came from. Take only as much as you need and never return leftover solutions to a reagent bottle. Discard leftovers in the proper container.
- Do not use your thumb as a stopper. Swirl gently or put a piece of paraffin over the opening to mix solutions.
- Label all test tubes and other containers with contents.
- Do not pour reagents and chemicals down the sink. Dispose of these only in designated containers.
- Put plant remains in the designated containers. Do not discard solids or plant materials down the sinks. They will clog up the sink. Use specified containers for such wastes.
- Be sure to use caution when using razor blades to prepare lab materials. Put the used blades into the designated container from which you obtained them. Do not leave them loose on the desks or counters. Never put razor blades in the wastepaper basket.
- Your instructor will review with you the location and, where applicable, use of the safety equipment in the laboratory including: emergency phone, first aid kit, fire extinguisher, eyewash.

3.2 Principle of Safety

Laboratory is not a place of playing or carrying out any careless activity therefore, the principle of safety is key. Anyone entering laboratory should be very careful and be conscious of what he/she has gone there to do. However, rules pertaining to safety can be extensive, different categories of concerns have been noted (Rashid, *et al.*, 2007).

3.2.1 Different Categories Where Safety Is Needed

i. General attitude
ii. Instrument operation
iii. Accidents
iv. Chemicals
v. Furnace, Ovens and Hot plates
vi. Handling gasses
vii. Maintenance
viii. Eating and drinking
ix. Protective Equipment
x. Waste disposals

General safety rules in different categories to ensure safety

1. General attitude
   i. Develop a positive attitude towards laboratory safety
   ii. Observe normal laboratory safety practices
   iii. Maintain a safe and clean work environment
   iv. Avoid working alone

2. Instrument operation
   i. Follow safety precautions provided by the manufacturer when operating instruments
   ii. Monitor instruments while they are in operation
   iii. Do not open centrifuge cover until machine has completely stopped
   iv. Atomic Absorption Spectrophotometer (AAS) must be vented to the atmosphere, ensure that the drain trap is filled with water before igniting the burner

3. Accidents
   i. Learn what to do in case of emergencies (fire, spill of chemicals, etc.), firefighting equipment must be readily accessible in the event of fire.
   ii. Learn emergency first aid. First aid supplies are a necessity and laboratory staff should be well trained in their use.
   iii. Immediately seek medical attention if affected by chemicals, use first aid until medical aid is available.
   iv. Access to eye-wash fountains and safety showers must not be locked. Fountain and showers should be regularly checked for proper operation.

4. Chemicals

i. Add acid to water and not water to acid when diluting the acid.
ii. Always put labels on bottles, vessels and wash-bottles containing reagents, solutions, samples and water.
iii. Handle per caloric acid and hazardous chemicals in fume hoods.
iv. With the wet oxidation method of sample digestion, destroy organic matter (OM) first with nitric acid.
v. Read the labels on the bottles before opening them.
vi. Wash hands after handling toxic/hazardous chemicals.
vii. Never suck the chemicals by mouth but use automatic pipetting devices.

5. Maintenance

i. All electrical, plumbing and instrument maintenance work should be done by qualified personnel.
ii. Fume hoods should be checked routinely .
iii. As most equipment operate on low wattage, use of Uninterrupted power supply (UPS) is necessary to provide stable power and allows the completion of any batch measurement in the event of power outage.

6. Eating and drinking

i. Do not eat, drink or smoke in the laboratory. This is essential both for reasons of health and to reduce contamination.
ii. Do not use laboratory glassware for eating/drinking.
iii. Do not store food in the laboratory.

7. Protective equipment

i. Use personal safety equipment as follow.
ii. **Body protection**: Use laboratory coat and chemical resistant apron.
iii. **Hand protection**: use gloves particularly when handling concentrated acids, bases and other hazardous chemicals.
iv. **Dust mask:** usually needed when grinding soil.
v. **Eye protection:** Use safety glasses with side shields. Persons wearing contact lens should always wear safety glasses in the laboratory. Make sure the people you work with know you are using contact lenses and it should not be worn around corrosives.
vi. **Full face shield**: wear face shields over safety glasses in experiments involving corrosive chemicals.
vii. **Foot protection:** proper foot wear should be used; sandals should be worn in the laboratories.

8. Waste disposal

i. Liquid waste should be poured carefully down the sink with sufficient water to dilute and flush it away.
ii. Dispose of chipped or broken glassware in specially marked containers.

3.2.2 Handling Contaminations

One of the most insidious enemies in any laboratory is contamination and therefore its sources must be identified and eliminated.

Some common sources of contaminations are as follow:

1. External dusts blown from the surrounding environment.
2. Internal dusts resulting from cleaning operations .
3. Cross- contaminations derived from handling many samples at the same time (e.g. handling plant and soil samples together).
4. Failure to store volatile reagents well away from the samples.
5. Washing materials especially soap powder.
6. Smoking in the laboratory.

4.0 CONCLUSION

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. Hazardous chemicals should be stored in plastic bottles. While working with chemicals, such as perchloric acid, a fume hood must be used. Chemicals must be labelled properly, indicating their hazardous nature. Bottles with inflammable substances need to be stored in stainless-steel containers. 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.

5.0 SUMMARY

You have learnt that safety precautions in the laboratory include but not limited to the following; Food and drink must not be allowed in lab unless food or drinks are provided as a part of the lab requirement for the intended

analysis. Even though lab tables and counters are wiped down before each lab set up, as a result of some laboratory exercises, chemical residues may be present on the tables. For labs exercises involving food or drinks, lab assistants and instructors will follow procedures that allow safe consumption. Smoking is totally not acceptable in all University buildings. Shoes must be worn in lab. If you have very long hair, please tie it back in the laboratory. Handle chemicals, reagents, and stains carefully and follow all warnings. All bottles and containers are labeled as to contents and potential hazards. If, for example, a label says avoid contact with substance and fumes, do so. For potentially hazardous chemicals, information on the hazards, proper handling, and clean-up is provided on Material Safety Data Sheets (MSDS). These are available in the lab. It is highly recommended that you spend the first few minutes of the lab consulting the MSDS. Read all precautions in the laboratory manual and on labels and follow directions exactly.

6.0 TUTOR-MARKED ASSIGNMENT

1. Why is safety a very important consideration in a soil science laboratory?
2. Mention 10 different categories where safety is needed in a laboratory.
3. Give at least (5) safety precautions in (5) of the categories mentioned above.
4. What are the sources of contaminations in the laboratory and how can they be handled?

7.0 REFERENCES/FURTHER READING

Rashid, R., Ryan, J., & Estefan, G. (2007). Soil and Plant analysis Laboratory Manual. International Center for Agricultural Research In The Dry Area National Agricultural Research Center, Islamabad, Pakistan
http://www.fao.org/3/a-i0131e.pdf

UNIT 2 PRINCIPLE OF QUALITY

CONTENTS

1.0 INTRODUCTION

The term "quality" has a relative meaning. This is expressed by the International Standard Organisation (ISO) definition: The totality of features and characteristics of a product or service that bear on its ability to satisfy stated or implied needs. In simpler words, one can say that a product has good quality when it complies with the requirements specified by the client. When projected on analytical work, quality can be defined as delivery of reliable information within an agreed span of time under agreed conditions, at agreed costs, and with necessary aftercare. The agreed conditions should include a specification as to the precision and accuracy of the data which is directly related to fitness of use and which may differ for different applications. Yet, in many cases the reliability of data is not questioned and the request for specifications omitted.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- know the meaning of safety as concerns laboratory activities
- understand the meaning of quality management, quality assurance and quality control.

3.0 MAIN CONTENT

3.1 Principle of Quality

Many laboratories work according to established methods and procedures which are not readily changed and have inherent default specifications. Moreover, not all future uses of the data and reports can be foreseen so that specifications about required precision and accuracy cannot even be given. Consequently, this aspect of quality is usually left to the discretion of the laboratory. However, all too often the embarrassing situation exists that a laboratory cannot evaluate and account for its quality simply because the necessary documentation is lacking. In the ensuing discussions numerous activities aimed at maintaining the production of quality are dealt with. In principle, three levels of organisation of these activities can be distinguished. From the top down these levels are:

1. Quality Management (QM)
2. Quality Assurance (QA)
3. Quality Control (QC)

3.1.1 Quality Management

Quality Management is the assembly and management of all activities aimed at the production of quality by organisations of various kinds. In the present case this implies the introduction and proper running of a "Quality System" in laboratories. A statement of objectives and policy to produce quality should be made for the organisation or department concerned (by the institute's directorate). This statement also identifies the internal organisation and responsibilities for the effective operation of the Quality System. Quality Management can be considered a somewhat wider interpretation of the concept of "Good Laboratory Practice" (GLP). Therefore, inevitably the basics of the present Guidelines largely coincide with those of GLP. Note. An even wider concept of quality management is presently coming into vogue: "Total Quality Management" (TQM). This concept includes additional aspects such as leadership style, ethics of the work, social aspects, relation to society, etc.

3.1.1.1 Good Laboratory Practice (GLP)

Quality Management in the present context can be considered a modem version of the hitherto much used concept "Good Laboratory Practice" (GLP) with a somewhat wider interpretation. The OECD Document defines GLP as follows: "Good Laboratory Practice (GLP) is concerned with the

organisational process and the conditions under which laboratory studies are planned, performed, monitored, recorded, and reported."
Thus, GLP prescribes a laboratory to work according to a system of procedures and protocols. This implies the organisation of the activities and the conditions under which these take place are controlled, reported and filed. GLP is a policy for all aspects of the laboratory which influence the quality of the analytical work. When properly applied, GLP should then:

- allow better laboratory management (including quality management)
- improve efficiency (thus reducing costs)
- minimise errors
- allow quality control (including tracking of errors and their cause)
- stimulate and motivate all personnel
- improve safety
- improve communication possibilities, both internally and externally.

The result of GLP is that the performance of a laboratory is improved and its working effectively controlled. An important aspect is also that the standards of quality are documented and can be demonstrated to authorities and clients. This results in an improved reputation for the laboratory (and for the institute as a whole). In short, the message is:

- say what you do
- do what you say
- do it better
- be able to show what you have done

The basic rule is that all relevant plans, activities, conditions and situations are recorded and that these records are safely filed and can be produced or retrieved when necessary. These aspects differ strongly in character and need to be attended to individually.

As an assembly, the involved documents constitute a so-called Quality Manual. This comprises then all relevant information on:

- Organisation and Personnel
- Facilities
- Equipment and Working materials
- Analytical or testing systems
- Quality control
- Reporting and filing of results.

Since institutions having a laboratory are of divergent natures, there is no standard format and each has to make its own Quality Manual. The present Guidelines contain examples of forms, protocols, procedures and artificial situations. They need at least to be adapted and many new ones will have to be made according to the specific needs, but all have to fulfill the basic requirement of usefulness and verifiability.

3.1.2 Quality Assurance

Proper Quality Management implies consequent implementation of the next level: Quality Assurance. The ISO definition reads: "the assembly of all planned and systematic actions necessary to provide adequate confidence that a product, process, or service will satisfy given quality requirements." The result of these actions aimed at the production of quality, should ideally be checked by someone independent of the work: the Quality Assurance Officer. If no QA officer is available, then usually the Head of Laboratory performs this job as part of his quality management task. In case of special projects, customers may require special quality assurance measures or a Quality Plan.

3.1.3 Quality Control

A major part of the quality assurance is the Quality Control defined by ISO as "the operational techniques and activities that are used to satisfy quality requirements. "An important part of the quality control is the Quality Assessment: the system of activities to verify if the quality control activities are effective, in other words: an evaluation of the products themselves. Quality control is primarily aimed at the prevention of errors. Yet, despite all efforts, it remains inevitable that errors are made. Therefore, the control system should have checks to detect them. When errors or mistakes are suspected or discovered it is essential that the "Five Ws" are trailed.

- what error was made?
- where was it made?
- when was it made?
- who made it?
- why was it made?

Only when all these questions are answered, proper action can be taken to correct the error and prevent the same mistake being repeated. The techniques and activities involved in Quality Control can be divided into four levels of operation:

1. First-line control: Instrument performance check.
2. Second-line control: Check of calibration or standardization.
3. Third-line control: Batch control (control sample, identity check).
4. Fourth-line control: Overall check (external checks: reference samples, inter-laboratory exchange programmes).

Because the first two control levels both apply to the correct functioning of the instruments they are often taken together and then only three levels are distinguished. This designation is used throughout the present Guidelines:

1. First-line control: Instrument check / calibration.
2. Second-line control: Batch control
3. Third-line control: External check

It will be clear that producing quality in the laboratory is a major enterprise requiring a continuous human effort and input of money. The rule-of-fist is that 10-20% of the total costs of analysis should be spent on quality control. Therefore, for quality work at least four conditions should be fulfilled:

- means are available (adequate personnel and facilities)
- efficient use of time and means (costs aspect)
- expertise is available (answering questions; aftercare)
- upholding and improving level of output (continuity)

In quality work, management aspects and technical aspects are inherently cobbled together and for a clear insight and proper functioning of the laboratory these aspects have to be broken down into their components. This is done in the ensuing chapters of this manual.

4.0 CONCLUSION

Quality control is the totality of features and characteristics of a product or service that bear on its ability to satisfy stated or implied needs. In simpler words, one can say that a product has good quality when it complies with the requirements specified by the client. When projected on analytical work, quality can be defined as delivery of reliable information within an agreed span of time under agreed conditions, at agreed costs, and with necessary aftercare.

5.0 SUMMARY

Proper Quality Management implies consequent implementation of the Quality Assurance. A major part of the quality assurance is the Quality Control defined by ISO as the operational techniques and activities that are used to satisfy quality requirements. An important part of the quality control is the Quality Assessment: the system of activities to verify if the quality control activities are effective, in other words: an evaluation of the products themselves.

6.0 TUTOR-MARKED ASSIGNMENT

1. Briefly explain three levels of organisation of quality in the laboratories.
2. Enumerate the benefits of good laboratory practice when properly applied.
3. What are the five Ws that are essential when errors or mistakes are suspected in the laboratories?
4. What are the four techniques involved in Quality Control levels of operation?
5. Outline the four conditions that must be fulfilled for quality works in the laboratory.

7.0 REFERENCES/FURTHER READING

http://www.fao.org/3/W7295E/w7295e07.htm#TopOfPage

UNIT 3 PRINCIPLE OF PRECISION AND ACCURACY

CONTENTS

1.0 INTRODUCTION

The issues of accuracy and precision are vital considerations for the analyst and can be determining factors in any method selection. Accuracy is the ability to obtain the "true" value and is dependent to a large degree on the availability and use of reliable standards (Jones 2001). Precision, on the other hand, is a measure of the degree of variability of an obtained result determined by repeated analyses of the same sample through all the steps from sample preparation to the final obtained result. Hislop (1980) has an article on the requirements for obtaining accurate and precise analytical results. Hurwitz (2000) also has evaluated various analytical procedures by assigning levels of performance based on years of use in the determination of elements and substances in a wide range of materials. Rayment*et al.*, (2000) looked at the percent coefficients of variation for a number of commonly performed soil analysis procedures, found that the pH determination had the lowest (<5%), that determinations for Cl, organic C, nitrate-N, DTPA Zn and Mn, and exchangeable Ca, K, and Mg ranged from 10 to 20%, that exchangeable Na was between 20 and 30%, and that Olsen P and Bray P were greater than 30%. They also looked at intra-laboratory RSDs (%), finding that the greatest precision was obtained for the determination of pH (RSD of 1.6%), that determinations for ammoniun-N, Bray P1, and organic carbon had RSDs of 6.2, 6.3, and 8.3%, respectively, and that nitrate-N (Cd reduction) and Olsen P had RSDs of 14.5 and 11.8%, respectively. All these evaluations would suggest that there may be an inherent variance in a particular method and/or that the methodology

associated with these assay procedures should be more carefully examined to determine the source of variance.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- understand the meaning of accuracy and precision in soil analytical procedures
- know what to do to ensure accuracy and precision in soil analytical technique.

3.0 MAIN CONTENT

3.1 Precision and Accuracy

Precision is defined as the agreement without conflict or 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 standardisation 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 “colored 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.

3.1.1 Precision/Repeatability/Reproducibility

Precision is a term that describes an instrument’s degree of freedom from random errors. If a large number of readings are taken of the same quantity by a high precision instrument, then the spread of readings will be very small. Precision is often, though incorrectly, confused with accuracy. High precision does not imply anything about measurement accuracy. A high precision instrument may have a low accuracy. Low accuracy measurements from a high precision instrument are normally caused by a bias in the measurements, which is removable by recalibration. The terms repeatability and reproducibility mean approximately the same but are applied in different contexts as given below.

Repeatability describes the closeness of output readings when the same input is applied repetitively over a short period of time, with the same measurement conditions, same instrument and observer, same location and same conditions of use maintained throughout. *Reproducibility* describes the closeness of output readings for the same input when there are changes in the method of measurement, observer, measuring instrument, location, conditions of use and time of measurement. Both terms thus describe the spread of output readings for the same input. This spread is referred to as repeatability if the measurement conditions are constant and as reproducibility if the measurement conditions vary. The degree of repeatability or reproducibility in measurements from an instrument is an alternative way of expressing its precision.

Figure 1) below shows the results of tests on three industrial robots that were programmed to place components at a particular point on a table. The target point was at the centre of the concentric circles shown, and the red x-marks represent the points where each robot actually deposited components at each attempt. Both the accuracy and precision of Robot 1) are shown to be low in this trial. Robot 2) consistently puts the component down at approximately the same place but this is the wrong point. Therefore, it has high precision but low accuracy. Finally, Robot 3) has both high precision and high accuracy, because it consistently places the component at the correct target position.

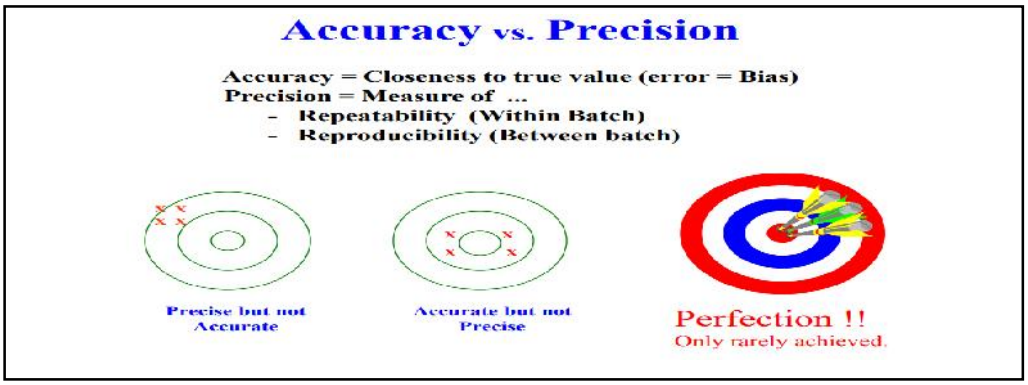

Fig. 1: Test of Accuracy and Precision

3.2 Errors

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, and the accuracy of the instruments and the skill of the technician are important factors.

Part of improving chemical analysis is to be able to identify the place where errors can occur and be able to evaluate their magnitude. There is possibility of making mistakes at any step of the analysis. These possible mistakes are called error. One way to find out if you have made mistake is to repeat the whole procedure for several replicate samples or replicates. The more the replicates, the higher the level of certainty of the results.

3.2.1 Sources of Errors

Errors could be classified based on the sources as:

1. *Personal error:* - These are errors that emanate from the person carrying out the analysis (the analyst). It could be due to lack of experience, dirty or unorganised work habit, lack of attention to details, or personal defect that may pose hindrance to accurate judgement (e.g. color blindness).
2. *Method errors:* - These are errors that emanate due to use of wrong method of analysis. Some methods may not be able to eliminate certain interferences in the course of analysis, thus leading to error.
3. *Instrumental errors:* These are errors resulting from improper functioning of the instrument used for the measurement. It could be due to improper calibration or low sensitivity of instrument.

Errors could also be classified based on the whether or not the magnitude can be ascertained and correction made or not. Errors originating from fixed case are referred to as determinate or

1. *systematic error*; while those originating from arbitrary or indeterminate processes are

2. *random errors.*

Measuring errors
When several runs of replicates are made on a sample, it is not likely that the results would be exactly the same. We can make use of the Arithmetic mean (average) with deviation from mean (absolute deviation, or relative deviation).

3.3 Detection Limits

In the analysis for elements, particularly trace elements in soils, plants and fertilisers 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.

4.0 CONCLUSION

Accuracy is the ability to obtain the “true” value and is dependent to a large degree on the availability and use of reliable standards. Precision, on the other hand, is a measure of the degree of variability of an obtained result determined by repeated analyses of the same sample through all the steps from sample preparation to the final obtained result.

5.0 SUMMARY

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, and the accuracy of the instruments and the skill of the technician are important factors.

6.0 TUTOR-MARKED/ASSIGNMENT

1. i. Define Precision and accuracy.
   ii. With the aid of diagram, should relationship between precision and accuracy
2. What is the relationship between precision, repeatability and reproducibility?
3. i. What do you understand by error in an analytical process?
   ii. State four sources of error.
4. Explain fully what you know about Detection Limits.

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UNIT 4 CALIBRATION AND USE OF BLANKSIN ANALYTICAL PROCEDURES

CONTENTS

1.0 INTRODUCTION

For any result of analytical procedure to be useful, meaningful and acceptable, quality is of utmost importance according to Standard Organisation Procedures (SOPs). Aspects considered include calibration, use of blanks performance characteristics of the procedure, and reporting of results. Virtually all activities associated with these aspects are geared towards one purpose which includes the production of reliable data with a minimum of errors. In addition, it must be ensured that reliable data are produced consistently. To achieve this appropriate programme of quality control *(QC)* must be executed or implemented. Quality control is the term used to describe the practical steps undertaken to ensure that errors in the analytical data are within the acceptable limit, appropriate for the use to which the data will be put. This therefore means that the errors (which are unavoidably made) have to be quantified to enable a decision whether they are of an acceptable magnitude, and that unacceptable errors are discovered so that corrective action can be taken. Therefore, quality control must detect both random and systematic errors. The procedures for *QC* primarily monitor the accuracy of the work by checking the bias of data with the help of (certified) reference samples and control samples and the precision by means of replicate analyses of test samples as well as of reference and/or control samples.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- understand the importance of calibrations and use of blanks in analytical procedure
- know how calibrations and blanks can be used while carrying out any laboratory analysis.

3.0 MAIN CONTENT

3.1 Calibration and Use of Blanks in Analytical Procedures

3.1.1 Calibration Graph

The construction and use of calibration graphs or curves in daily practice of a laboratory is of high necessity. Calibration of instruments (including *adjustment*) is also referred to as *standardisation.* The confusion about these terms is mainly in word usage and the terms calibration curve and standard curve are always used interchangeably. The term "curve" implies that the line is not straight. However, the best calibration lines are linear (straight) and, therefore, the general term "graph" is preferred. For many measuring techniques calibration graphs have to be constructed. The technique is simple and consists of plotting the instrument response against a series of samples with known concentrations of the analyte (standards). In practice, these standards are usually pure chemicals dispersed in a matrix corresponding with that of the test samples (the "unknowns"). By convention, the calibration graph is always plotted with the concentration of the standards on the x-axis and the reading of the instrument response on the *y-axis.* The unknowns are determined by interpolation, not by extrapolation, so that a suitable working range for the standards must be selected. In addition, in the present discussion it is assumed that the working range is limited to the linear range of the calibration graphs and that the standard deviation does not change over the range. Non-linear graphs can sometimes be linearised in a simple way, e.g. by using a log scale (in potentiometer), but usually imply statistical problems (polynomial regression) for which the reader is referred to the relevant literature. It should be mentioned, however, that in modem instruments which make and use calibration graphs automatically these aspects sometimes go by unnoticed.

3.1.1.1 How to Obtain Calibration Graphs Are

1. **Standards are made in a solution with the same composition as the Extractants :** The standards are made in a solution with the same composition as the extractant used for the samples (with the same dilution factor) so that all measurements are done in the same matrix. This technique is often practiced when analysing many batches where the same standards are used for some time. In this way an incorrectly prepared extractant or matrix may be detected (in blank or control sample).
2. **The standards are made in the blank extract**. A disadvantage of this technique is that for each batch the standards have to be pipetted. Therefore, this type of calibration is sometimes favored when only one or few batches are analysed or when the extractant is unstable. A seeming advantage is that the blank can be forced to zero. However, an incorrect extractant would then more easily go by undetected. The disadvantage of pipetting does not apply in case of automatic dispensing of reagents when equal volumes of different concentration are added (e.g. with flow-injection).
3. **Standard additions technique is less common** but useful in special cases is the so-called *standard additions* technique. This can be practiced when a matrix mismatch between samples and standards needs to be avoided: the standards are prepared from actual samples. The general procedure is to take a number of aliquots of sample or extract, add different quantities of the analyte to each aliquot *(spiking)* and dilute to the final volume. One aliquot is used without the addition of the analyte (blank). Thus, a standard series is obtained.

If calibration is involved in an analytical procedure, the SOP for this should include a description of the calibration sub-procedure. If applicable, including an optimalisation procedure (usually given in the instruction manual).

3.1.1.2 Construction and Use

In several laboratories calibration graphs for some analyses are still adequately plotted manually and the straight line (or sometimes a curved line) is drawn with a visual "best fit", e.g. for flame atomic emission spectrometry, or colorimetric. However, this practice is only legitimate when the random errors in the measurements of the standards are small: when the scattering is appreciable the line-fitting becomes subjective and

unreliable. Therefore, if a calibration graph is not made automatically by a microprocessor of the instrument, the following more objective and also quantitatively more informative procedure is generally favored.
The proper way of constructing the graph is essentially the performance of a regression analysis i.e., the statistical establishment of a linear relationship between concentration of the analyte and the instrument response using at least six points. This regression analysis (of reading *y* on concentration *x*) yields a correlation coefficient *r* as a measure for the fit of the points to a straight line (by means of *Least Squares).*

3.1.1.3 Independent Standards

It cannot be overemphasised that for quality control (*QC),* a calibration should always include measurement of an *independent standard* or *calibration verification standard* at about the middle of the calibration range. If the result of this measurement deviates so much from the correct or expected value (say > 5%), then inspection is indicated. Such an independent standard can be obtained in several ways. Most usually it is prepared from pure chemicals by another person than the one who prepared the actual standards. Obviously, it should never be derived from the same stock or source as the actual standards. If necessary, a bottle from another laboratory could be borrowed. In addition, when new standards are prepared, the remainder of the old ones always have to be measured as a mutual check (include this in the SOP for the preparation of standards!).

3.1.1.4 Measuring a Batch

After calibration of the instrument for the analyte, a batch of test samples is measured. Ideally, the response of the instrument should not change during measurement *(drift* or *shift).* In practice this is usually the case for only a limited period of time or number of measurements and regular recalibration is necessary.

The frequency of recalibration during measurement may vary widely depending on the following:

- Technique
- Instrument
- Analyte
- Solvent
- Temperature and
- Humidity

In general, emission and atomising techniques (especially while using Atomic Absorption Spectrophotometer) are more sensitive to drift (or even sudden shift: by clogging) than colorimetric techniques. Also, the techniques of recalibration and possible subsequent action vary widely.

3.1.2 Blanks

A blank or blank determination is an analysis of a sample without the analyte or attribute, or an analysis without a sample, i.e. going through all steps of the procedure with the reagents only. The latter type is the most common as samples without the analyte or attribute are often not available or do not exist. Another type of blank is the one used for calibration of instruments. Thus, we may have two types of blank within one analytical method or system:

- a blank for the whole method or system and
- a blank for analytical sub-procedures (measurements) as part of the whole procedure or system.

For instance, in the cation exchange capacity (CEC) determination of soils with the percolation method, two methods or system blanks are included in each batch: two percolation tubes with cotton wool or filter pulp and sand or celite, but without sample. For the determination of the index caution (NH_4 by colorimetric or Na by flame emission spectroscopy) a blank is included in the determination of the calibration graph. If NH_4 is determined by distillation and subsequent titration, a blank titration is carried out for correction of test sample readings.

The Importance of blanks

1. In many analyses sample results are calculated by subtracting blank readings from sample readings.
2. Blank readings can be excellent monitors in quality control of reagents, analytical processes, and proficiency.
3. They can be used to estimate several types of method detection limits.

   For blanks the same rule applies as for replicate analyses: the larger the number, the greater the confidence in the mean. The widely accepted rule in routine analysis is that each batch should include at least two blanks. For special studies where individual results are critical, more blanks per batch may be required (up to eight). To ensure quality, control charts are made of blank readings identically to those of control samples. The between-batch variability of the blank is expressed by the standard deviation calculated from

the *control chart of the Mean of Blanks;* the precision can be estimated from the *Control Chart of the Range of Duplicates of Blanks.*

In many laboratories, no control charts are made for blanks. Sometimes, analysts argue that 'there is never a problem with my blank; the reading is always close to zero'. Admittedly, some analyses are more prone to blank errors than others. This, however, is not a valid argument for not keeping control charts. They are made to monitor procedures and to alarm when these are out of control (shift) or tend to become out of control (drift). This can happen in any procedure in any laboratory at any time.

From the foregoing discussion it will be clear that signals of blank analyses generally are not zero. In fact, blanks may found to be negative. This may point to an error in the procedure: e.g. for the zeroing of the instrument an incorrect or a contaminated solution was used or the calibration graph was not linear. It may also be due to the matrix of the solution (e.g. extractant), and is then often unavoidable. For convenience, some analysts practice "forcing the blank to zero" by adjusting the instrument. Some instruments even invite or compel analysts to do so. This is equivalent to subtracting the blank value from the values of the standards before plotting the calibration graph. From the standpoint of Quality Control this practice must be discouraged. If zeroing of the instrument is necessary, the use of pure water for this is preferred. However, such general considerations may be overruled by specific instrument or method instructions. This is becoming more and more common practice with modem sophisticated hi-tech instruments. Whatever the case, a decision on how to deal with blanks must made for each procedure and laid down in the SOP concerned.

4.0 CONCLUSION

Calibration of instruments (including *adjustment*) is also referred to as *standardisation.* For many measuring techniques calibration graphs have to be constructed. The technique is simple and consists of plotting the instrument response against a series of samples with known concentrations of the analyte (standards). In practice, these standards are usually pure chemicals dispersed in a matrix corresponding with that of the test samples (the "unknowns"). Also, a blank determination is an analysis of a sample without the analyte or attribute, or an analysis without a sample, i.e. going through all steps of the procedure with the reagents only.

5.0 SUMMARY

By convention, the calibration graph is always plotted with the concentration of the standards on the x-axis and the reading of the instrument response on the *y-axis.* The unknowns are determined by interpolation, not by extrapolation, so that a suitable working range for the standards must be selected. In addition, in the present discussion it is assumed that the working range is limited to the linear range of the calibration graphs and that the standard deviation does not change over the range. A blank or blank determination is an analysis of a sample without the analyte or attribute, or an analysis without a sample, i.e. going through all steps of the procedure with the reagents only.

6.0 TUTOR-MARKED ASSIGNMENT

1. Explain fully what you understand by calibration and blank in analytical procedure.
2. State how calibration graph can be obtained.
3. Briefly explain the following terms as relates to calibration graph
   i. Independent standards
   ii. Construction and use
   iii. Measuring a batch.
4. What are the factors that may affect the frequency of recalibration during measurement?
5. State the importance of blank in analytical procedure.

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MODULE 2 ANIMAL, CROP AND SOIL SAMPLES ANALYSIS

UNIT 1 ANIMAL FEED SAMPLE ANALYSIS

CONTENTS

1.0 INTRODUCTION

Analysis of animal feed is of very vital importance since its nutritional content and value determines the health and quality of our animals, which invariably determines the health and nutritional quality of man who depends on animals as major source of protein. Types of analyses conducted by laboratory are proximate analyses, macro-minerals, micro-minerals at trace level, chromatographic analyses (such as amino acids, fatty acids, etc.) and chromatographic analyses at trace level (contaminants such as aflatoxins, pesticides and pesticide residues, antibiotics, etc.) (De Jonge and Jackson 2005). Several standard and laboratory methods have been developed over the years for the detection of both nutrients and contaminants in feed ingredients and feedstuffs.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- know different methods of analysis used for animal feed
- sample animal feed for laboratory analysis.

3.0 MAIN CONTENT

3.1 Methods of Analysis in the Feed Industry

Malomo and Ihegwuagu(2017)classified the methods into official methods (required by law and used by regulatory and complying organization), reference methods (developed by collaborating organizations for validation purposes), screening or rapid methods (usually for large samples to determine whether further analysis are required with more accurate methods), routine methods (can be official, standard or modified methods used for routine testing), automated methods (may be official or screening methods that adopts automated equipment), and modified methods (usually official or standard methods, which have been modified to make it simple and applicable to wide range of samples).In the absence of standardized analytical methods, laboratory methods that meet certain criteria, validated and accredited in line with international guidelines and quality assurance protocols, may serve as alternatives. 'Accuracy, applicability (matrix and concentration range), limit of detection, limit of determination, precision, repeatability and reproducibility' are some of the criteria that laboratory methods must meet to serve as an alternative to standard methods.

Table 1: Description of typical tests in feed analyses

Parameter	Description
Dry mater	Part of the sample that remains after dying at 103°C
Crude ash	Part of the sample that remains after incineration at 550°C
Ash insoluble in acid (sand)	Ash that remains after boiling in strong acid
Crude protein	Total nitrogen content and to calculate the protein content by multiplying the nitrogen content by an appropriate conversion factor (usually ×6.25). Kjeldahl method (Nitrogen is converted into ammonia which is absorbed in boric acid and titrated against a standard acid); Dumas method (With complete combustion of sample at950°C in the presence of oxygen, nitrogen is converted to a gaseous state and reduced toN2, followed by measurement in a thermal conductivity cell)
Crude fat	Non-polar extractable fraction of the sample. The extraction can be performed with or without prior acid hydrolysis, both being complementary methods. The laboratory should offer both options
Fibre analysis	Digestion of feed directly in the detergent solution and filtration *using crucibles* (official standard method).

	Digestion of sample whilst in a *nylon bag* and then washing the bag containing the digested sample to make it detergent free.
Starch	Starch can be measured by the classical Ewers method or with an enzymatic method. The enzymatic method can be used for all sample types and is therefore preferable.
Gross energy	Gross energy represents the total energy value of the sample and is measured by bombcalorimeter.
Minerals	Minerals are generally measured by spectrometric methods following incineration and hydrolysis.
Amino acids (excluding tryptophan)	The standard method for the determination of amino acids is based on the hydrolysis of protein to amino acids using a strong acid with or without previous oxidation, followed bychromatographic separation and detection after derivatisation
Amino acids (tryptophan)	Determination of tryptophan is based on an alkaline hydrolysis followed bychromatographic Separation
Fatty acids	The standard method for fatty acids is based on isolation and derivatisation, followed by gas chromatographic separation
Vitamins	Determination of individual vitamins is based on extraction, followed by clean-up, concentration if needed, and chromatographic measurement.
Reducing sugar	Reducing sugars contain the most important sugars, including glucose, fructose and sucrose. Determination is based on the Luff-School principle.
Mycotoxins	Mycotoxins are undesirable substances produced by fungi (moulds). These present apotential danger to animal and human health. The maximum levels are nationally and internationally regulated. The different methods are based on extraction, purification, chromatographic separation and detection.
Pesticides	Pesticides are undesirable substances whose maximum levels are defined in national and international regulations. These regulations demand a low detection limit and positive identification of the pesticides, which is achieved by using mass spectrometric detection. The methods are based on extraction, purification, derivatisation, chromatographic separation and identification.

Source: De Jonge and Jackson (2005)

3.1.1 Proximate Analyses

Characterization of feeds and feed ingredients for general nutritional parameters are done using proximate analyses. The ability to conduct proximate analyses is the minimum requirement for laboratories (De Jonge and Jackson 2005). Proximate analyses can be conducted in any basic nutrition laboratory while other analyses can be done in more complex laboratories. Analytical methods for proximate composition and some other feed components are presented in Table 1.

3.1.2 Risk Analysis

Demands for higher standards in all aspects of feed production have been on the increase globally. This may be in part due to the increasing awareness of the role of feeds in potential hazards associated with food of animal origin. Accordingly, appropriate codes have been developed by relevant international bodies to assist national authorities to take measures that would mitigate most of these risks, particularly those of public health importance and which may constitute barriers to international trades. Risk analysis is an objective and defensible mechanisms for risks reduction that are associated with health and other factors. For example, Article 2.1 of the aquatic animal health code, which addresses animal health issues in international trades, provided basic guide and steps for import risk analysis in relation to aquatic animals and aquatic animal products (OIE, 2014). However, the principles and methods of risk analysis are the same for both aquatic and terrestrial animals and products, including feedstuff. The four components involved with risk analysis are highlighted below:

a. **Hazard identification:** This is a categorization step in the risk analysis and the risk assessment should be concluded at this stage in the absence of any identified potential risk.
b. **Risk assessment:** Involves both qualitative and quantitative methods of risk assessment, each with its relevant outputs. The steps are entry assessment; exposure assessment (both entry and exposure assessment steps involve the assessment of biological, country and commodity factors); consequence assessment (direct and indirect consequences); and risks estimation which integrates results of the entry, exposure and consequence assessments to produce the overall measures of risks associated with the hazard identified at the outset. The risk assessment should be concluded at either entry assessment or exposure assessment step if no substantial risk is demonstrated. The whole risks pathway from identified hazard to unwanted outcome is taken into account by the risk estimation step.

c. **Risk management:** This involves deciding and implementing protective measures and at the same time minimising the negative effects on trade. Components of risk management include risk evaluation, option evaluation, implementation, and monitoring and review.
d. **Risk communication:** This requires having a risk communication strategy in place at the outset of each risk analysis.

3.1.3 Quality Assurance and Control in Feed Analysis

There are variations in the results of feed analyses obtained from different laboratories and these have been a major source of concern in the feed industry and among relevant authorities globally (Key *et al.*, 1997).Efforts to limit unacceptably high variations in the results of analysed samples in various laboratories, which are sometimes difficult to attribute to genotypic, environmental or inter laboratory differences, contributed to the development of quality assurance and control for analysis (Mueller-Harvey 2004). Use of quality assurance schemes, inter-laboratory evaluation programmes and reference materials were recommended by Petterson *et al.*, (1999) to reduce errors due to laboratory and methodological differences. Laboratory quality assurance scheme requires the implementation of management quality policy statement, objectives of the scheme, control of samples and records, equipment maintenance, methods evaluation, measurement principles, training, methods selection, intra- and inter-laboratory testing, reference standards, field and lab sampling, statistical considerations, audits, corrective actions, programme revisions and update. These could be grouped properly under the four guiding principles of valid analytical measurement (VAM), which was developed in 1994 in the United Kingdom by the Department of Trade and Industry to contribute to validity of analytical data, namely (Malomo and Ihegwuagu 2017):

i. Use of properly validated methods of measurement.
ii. Incorporate certified reference materials (CRMs) in quality assurance protocols to ensure traceability measurements.
iii. Independent assessment of laboratory's performance for particular tests through participation in national and international proficiency testing schemes (PTS).
iv. Independent approval of quality assurance arrangements of laboratories by accreditation or licensing to a recognised quality standard.

3.1.4 Some Aspects of and Considerations in Feed Sampling

The accuracy and reliability of the results of any analysis in the animal feed industry begins with the quality of sampling. An analysis can be said to be as good as its sampling because several challenges that can affect accuracy and reliability of the results are associated with sampling of the feeds and feed materials. It is, therefore, critical to ensure sampling of animal feed ingredients and feeds is done in an area and in a way that makes the procedures easy, as well as minimise the risk of contamination and cross contamination, makes proper performance of the laboratory analysis possible, and ensures all safety and health precautions for the sampler and the environment.

3.1.5 Types of samples

Pierce (1994) and Malomo and Ihegwuagu (2017) identified various types of samples depending on their purposes and uses as follows:

i. check sample
ii. composite sample
iii. discrete sample
iv. duplicate sample
v. official sample
vi. purchasing sample
vii. referee sample
viii. reference sample
ix. retained sample
x. standard sample
xi. working sample.

3.1.6 Sampling Errors

Sampling errors may be due to the heterogeneity of the inspected characteristics, the random nature of sampling, and the known and acceptable characteristics of the sampling plan (FAO, 2004). Some of the measures to be taken to minimise sampling errors in the animal feed industry include.

i. **Sampling procedures should be based on the objectives, standards, or purposes of the analysis.** Simple random sampling, stratified random sampling, and systematic sampling are examples of common sampling schemes used in the feed industry.

ii. **Use appropriate sampling equipment that will not introduce contamination.** For example, do not use lead containing materials to collect samples meant for lead analysis. Examples of sampling equipment include grain probes (slotted grain probes, open-handled grain probes, open-handled spiral probe); pelican grain sampler; tapered bag triers; double tube bag triers; single-tube, open-ended bag triers; bomb or zone sampler.
iii. **Collect representative samples.** If the samples collected are not representative of the whole, the results of the analysis become skewed. To collect a representative sample, the sampling scheme must be followed, adequate quantity of sample must be collected, and sampling equipment and procedure must be appropriate, required inspection of sample, among other things
iv. **Use the right quantity of materials** and avoid splashing of samples during collection and analysis. Several errors can be associated with the splitting of samples, if not done carefully.
v. Use standard reference materials.
vi. Repeat analysis.
vii. Validate laboratory methodologies and use standard methods.
viii. Use well trained and knowledgeable personnel.
ix. Observe sampling precautions required for the methods of analysis.
x. Use the appropriate sampling plans.

3.1.7 Sampling Plans Selection

Sampling plan is a planned procedure that enables the choice of separate samples from a lot, for the purpose of getting the needed information, such as a decision on compliance status of a lot. It is also a scheme that defines the number of items to collect and the number of non-conforming items required in a sample to evaluate the compliance status of a lot. Thus, without an appropriate sampling plan, it may be practically impossible to accurately decide the compliance status of a particular lot of a product. Codex guideline for sampling recommends seven important considerations in selecting appropriate sampling plans incompliance with relevant standards in the feed industry (FAO 2004):

(i) Existence (or not) of international reference document on sampling of the products under consideration;
(ii) Nature of control (individual or whole lot),
(iii) Nature of the characteristic to control (qualitative or quantitative characteristics),

(iv) Choice of the quality level, limiting quality or acceptance quality level, in line with principles laid down in codex manual of procedures and the type of risk,
(v) Nature of the lot, that is bulk or pre-packed products, size, homogeneity and distribution concerning the characteristics of control,
(vi) Composition of sample, that is those composed of single or more than one sampling unit,
(vii) Choice of the type of sampling plan.

3.1.8 Preparation of Samples

FAO (2004) also sets the guidelines for sample preparation. A primary sample is prepared by direct collection of items or incremental samples. During the first stage of the sampling process, primary samples are collected from lots of items or incremental samples for pre-packed or bulk feeds, respectively. In order to facilitate laboratory analysis, sufficient quantity of the primary samples of similar size should be collected. Necessary precautions must be taken to ensure sample integrity and avoid any form of contamination throughout the entire process of sampling and analysis. Composite sample is prepared, whenever required by the sampling plan, by carefully mixing the primary samples. This involves primary samples collected from a lot of pre-packaged products or incremental samples from a bulk (not-pre-packed) lot. In composite sample preparation, combination of primary samples may lead to loss of information on sample-to-sample variation. The composite sample should, except when too large, constitute the final sample which is sent to the laboratory for analysis.

4.0 CONCLUSION

The reliability and quality of the analysis depends on the accuracy of sampling. However, adequate care must be taken to ensure that the analytes are handled in a way that will prevent degradation and errors. Laboratory quality assurance scheme requires the implementation of management quality policy statement, objectives of the scheme, control of samples and records, equipment maintenance, methods evaluation, measurement principles, training, methods selection, intra- and inter-laboratory testing, reference standards, field and lab sampling, statistical considerations, audits, corrective actions, programme revisions and update to ensure quality and reliable results of laboratory feed analysis.

5.0 SUMMARY

Feed sampling and analyses are essential parts of the processes which ensure that feed stuffs and the resultant food animals meet all necessary standards. Where a feed which has been identified as not satisfying the feed safety requirement is part of a batch, lot or consignment of feed of the same class or description, it shall be presumed that all of the feed in that batch, lot or consignment is so affected, unless following a detailed assessment there is no evidence that the rest of the batch, lot or consignment fails to satisfy the feed safety requirement. This is an important point if you get an adverse sample result when sampling.

6.0 TUTOR-MARKED ASSIGNMENT

1. In a tabular form mention 10 feed parameters and describe briefly their laboratory procedures.
2. Explain the term proximate analysis in laboratories.
3. What do you understand by risk analysis? State four components involved with risk analysis.
4. List 10 types of samples you studied.
5. Define Sampling error as given by FAO and state 10 steps to be taken to minimise sampling error in animal feed analysis.
6. What do you understand by sampling plan? What are the considerations while selecting a sampling pan?

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UNIT 2 PLANT SAMPLING AND SAMPLE PREPARATION FOR ANALYSIS

CONTENTS

1.0 INTRODUCTION

Plant and soil testing enable 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). Sample collection is critical for plant analysis as plant nutrient composition varies with age, the portion of the plant sampled, and many other factors. Mistakes or carelessness in selecting, collecting, handling, preparing, or shipping plant tissue for analysis can result in unreliable data, which may lead to incorrect interpretations and recommendations. Standards, against which the sample is evaluated, have been selected to represent the plant part and time of sampling that best define the relationship between nutrient composition and plant growth. Deviation from the prescribed protocol severely limits this interpretations capability. It is, therefore, critical to follow a standard sampling procedure. However, when plant analysis is being used to confirm a suspected nutrient deficiency, the samples should be taken as early in the season as possible so that the deficiency can be corrected and minimize the potential yield loss. Plants showing abnormalities usually continue to accumulate nutrients even if growth is impaired by some limiting factor.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- understand the meaning of crop sampling and sample preparation
- carry out crop sampling and sample preparation.

3.0 MAIN CONTENT

3.1 Crop Sampling and Sample Preparation

3.1.1 Crop Sampling

Crop sampling is crucial for crop tissue analysis to enable researchers in the field of crop science carry out sustainable research works. This will lead to adequate crop improvements, genetic enhancement and modifications as well as improved productivity. Crop sampling has to be done with utmost carefulness as any mistakes or carelessness in selecting, collecting, handling, preparing, or shipping plant tissue for analysis can result in unreliable data, which may lead to incorrect interpretations and recommendations. Samples should not be taken from plants that obviously have been stressed from causes other than nutrients. Do not take samples from plants that are dead or insect damaged; Are mechanically or chemically injured; Have been stressed by too much or too little moisture (i.e., flooding or drought); Have been stressed by abnormally high or abnormally low temperature. Sample normal and abnormal areas when a nutrient deficiency is suspected (even without visual symptoms), or there is a need to compare different areas in a field, it is recommended that similar plant parts be collected separately from both the affected plants and adjacent normal plants that are at the same stage of growth. In this way, a better evaluation can be made between the nutritional status of healthy and abnormal plants of the same variety grown under the same conditions.

3.1.1.1 Collection and Treatment of Plant Material

Select plant material with the desired or expected composition. Realise that the composition of different parts of a plant (leaf, stem, flower, fruit) may differ considerably and that, in general, the control sample should match the test samples as much as possible. If the fresh material is contaminated (e.g. by soil, salts, dust) it needs to be washed with tap water or dilute (0.1 M*)* hydrochloric acid followed by deionised water. For test samples, to minimise the change of concentration of components, this washing should be done in a minimum of time, say within half a minute. For the preparation of a control sample this is less critical.
The sample is dried at 70°C in a ventilated drying oven for 24 hours. The sample is then cut and ground to pass a 1 mm sieve. Storage can be done as described for soil samples. *Note.* During the pretreatment (drying, milling, sieving) both soil and plant material may be contaminated by the tools used. In this way the concentration of certain elements (Cu, Fe, Al, etc.,)

may be increased. Like the washing procedure, this problem is less critical for control samples than for test samples (unless the contamination is present as large particles).

Precautions during crop sampling
The following guidelines have to be followed during sampling as serious error may occur which may lead to grave consequences. Such precautions are as follow:

1. Samples should not be taken from plants that obviously have been stressed from causes other than nutrients.
2. Do not take samples from plants that are dead or insect damaged
3. Are mechanically or chemically injured
4. Have been stressed by too much or too little moisture (i.e., flooding or drought);
5. Have been stressed by abnormally high or abnormally low temperature.
6. Sample normal and abnormal areas when a nutrient deficiency is suspected (even without visual symptoms), or there is a need to compare different areas in a field, it is recommended that similar plant parts be collected separately from both the affected plants and adjacent normal plants that are at the same stage of growth.

3.1.2 Plant Tissue Sample Preparation

- After a plant sample has been collected, it should be prepared for shipment or delivery to the laboratory.
- Roots or foreign material attached to the sample should be removed and discarded. Plant tissue must then be dusted off to remove soil particles.
- DO NOT WASH tissue since soluble nutrients will be leached out of the sample. If tissue is to be mailed, the sample should be air-dried above a heating vent or in the sun for one to two days to avoid mold formation during shipment.
- Place the plant sample in a paper bag in a large paper envelope for shipment. Do not pack the sample tightly into the mailing container or put samples in plastic or polyethylene bags as this will also promote mold development.
- Plant samples that are delivered to the laboratory do not need to be air-dried if they are delivered within one day after sampling. Samples to be delivered directly to the laboratory at a later date may be kept frozen or air-dried until they are delivered.

- Include Soil Sample Soil test results for pH, organic matter, phosphorus, and potassium (routine test) can be useful for correlating with plant analysis results to pinpoint a nutrient problem.
- A composite soil sample, consisting of five or more cores, taken to a depth of 6-7 inches, should be taken from the same area where the plant sample was collected. For row crops, avoid the fertilizer band by sampling from the middle of the row.
- Put the sample into a soil sample bag or other waterproof container and label the soil sample with the same field and sample number as that assigned to the tissue sample.
- Package corresponding plant and soil samples together, but make certain soil sample bags do not open in transit as spilled soil will contaminate plants.

Precautions during Plant tissue sample preparation

1. Roots or foreign material attached to the sample should be removed and discarded.
2. Plant tissue must then be dusted off to remove soil particles.
3. DO NOT WASH tissue since soluble nutrients will be leached out of the sample.
4. If tissue is to be mailed, the sample should be air-dried above a heating vent or in the sun for one to two days to avoid mold formation during shipment.
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7. Plant samples that are delivered to the laboratory do not need to be air-dried if they are delivered within one day after sampling.
8. Samples to be delivered directly to the laboratory at a later date may be kept frozen or air-dried until they are delivered.
9. Include Soil Sample Soil test results for pH, organic matter, phosphorus, and potassium (routine test) can be useful for correlating with plant analysis results to pinpoint a nutrient problem.
10. A composite soil sample, consisting of five or more cores, taken to a depth of 6-7 inches, should be taken from the same area where the plant sample was collected.

3.1.3 What to Do with Samples

A “Plant Analysis Information Sheet” should be filled out for any samples submitted. Use a separate information sheet for each sample. Plant samples, corresponding soil samples, and accompanying information sheets can be obtained and turned in at the County Extension Office especially in the United States. Samples may also be sent or delivered to the laboratory directly. The University of Wisconsin laboratory that conducts the plant analysis program is the Soil and Plant Analysis Laboratory at Madison. Some, but not all, private laboratories also analyse plant tissues; therefore, you should check with your laboratory on the specific services they provide before submitting the samples.

3.1.4 What the Analysis Report Will Include

The report will show the concentration of N, P, K, Ca, Mg, S, Zn, Mn, B, Cu, Fe, Al, and Na in the plant sample. If a soil was submitted with the plant sample, soil analyses for pH, organic matter, P, K, and any special soil test results will also be reported. In addition, the analytical levels of nutrients in the plant and soil will be interpreted to reflect nutrient deficiencies, toxicities, or imbalances by the sufficiency range approach, and if calibration data are available, the nutrient ratio method. When warranted, fertiliser recommendations will be made based on the analytical results. Most commonly grown field vegetables and fruit crops will receive these interpretations and recommendations. For those plant materials where calibration data are not available, these analytical results will be provided without interpretation.

3.1.5 Procedure for Plant Preparation for Analysis

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.

3.1.6 Parts of Crops Analysed

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 1 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 2 presents some critical values for a range of crops.

Table 2: General sufficiency or optimal range of nutrients in plants

Nutrients	Sufficiency or optimal level

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

FAO, 2008

Table 3: Crop Part to be sampled, with age or growth stage

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 tasseling
Barley Flag	leaf at head emergence
Pulse	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	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)
Onions	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, midsummer 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	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

FAO, 2008

4.0 CONCLUSION

You have learnt that samples should not be taken from plants that obviously have been stressed from causes other than nutrients. Do not take samples from plants that are dead or insect damaged; Are mechanically or chemically injured; Have been stressed by too much or too little moisture (i.e., flooding or drought); Have been stressed by abnormally high or abnormally low temperature. Sample normal and abnormal areas when a nutrient deficiency is suspected.

5.0 SUMMARY

The analytical levels of nutrients in the plant and soil will be interpreted to reflect nutrient deficiencies, toxicities, or imbalances by the sufficiency range approach, and if calibration data are available, the nutrient ratio method. When warranted, fertilizer recommendations will be made based on the analytical results. Most commonly grown field vegetables and fruit crops will receive these interpretations and recommendations. For those plant materials where calibration data are not available, these analytical results will be provided without interpretation.

6.0 TUTOR-MARKED ASSIGNMENT

1. What is plant sampling and why is it crucial in plant analysis?
2. What are the precautions to take while carrying out crop sampling?
3. State 10 precautions during plant tissue sample preparation.
4. Mention at least ten (10) parameters to be measured during plant analysis.
5. Discuss what to do with samples after collection.
6. Outline the procedure for plant preparation for analysis.

7.0 REFERENCES/FURTHER READING

FAO (2008) Guides to Laboratory Establishment for Plant Nutrient Analysis. Fertilizer and Plant nutrition Bulletin Vol. 19. Rome.

http://www.soils.wisc.edu/extension/pubs/pa_sampling.pdf

UNIT 3 SOIL SAMPLING AND SAMPLE PREPARATION

CONTENTS

1.0 INTRODUCTION

Soil sampling is a method that can be used for exploration of soil resources that lack obvious surface manifestations. Soils that are above or adjacent to a “hidden” system will have a unique chemistry that can be indicative of such system at depth and a zone of relatively high permeability. 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.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- know the meaning of soil sampling and sample preparation
- understand the meaning of crop sampling and sample preparation.

- know the basic procedures, techniques and equipment's used in soil and crop sampling and sample preparations

3.0 MAIN CONTENT

3.1 Soil Sampling and Sample Preparation

3.1.1 Soil Sampling

The major objectives of soil sampling have been to determine the average nutrient status of a field and to provide some measure of nutrient variability in a field. Soil sampling for precision agriculture is the trending dimension of agricultural researches and has these same objectives with some modifications. Instead of a field, producers are interested in areas within fields. They also are interested in relating trends in soil fertilizer levels to other field properties that are predictable or easily measured. Knowledge of factors influencing soil nutrient levels including soil type, topography, cropping history, manure application, fertilizer application and leveling for irrigation will help the producer determine the most effective sampling approach. The basic principles of soil sampling still apply to precision sampling. An adequate number of samples should be collected to accurately characterise nutrient levels. The samples should be collected to the proper depth for non-mobile and mobile nutrients. Samples should be handled and stored to minimise contamination and degradation.

3.1.1.1 Collection and Treatment of Soil Material

The amount of material to be collected depends on the turn-over of the sample material, the expected stability and the amount that can be handled during preparation. Thus, amounts may range from a few kilos to a hundred kilo or more. The material is collected in plastic bags and spread out on plastic foil or in large plastic trays in the institute for air-drying (do not expose to direct sunlight; forced drying up to 40°C is permitted). Remove large plant residues. After drying, pass the sample through a 2 mm sieve. Clods, not passing through the sieve are carefully crushed (not ground!) by a pestle and mortar or in a mechanical breaker. Gravel, rock fragments etc. not passing through the sieve are removed and discarded. The material passing through the sieve is collected in a bin or vessel for mechanical homogenisation. If the whole sample has to be ground to a finer particle size this can be done at this stage. If only a part has to be ground finer, this should be done after homogenisation. Homogenisation may be done with a shovel or any other instrument suitable for this purpose. Some laboratories use a concrete mixer. Mixing should be intensive and complete. After that,

the bulk sample is divided into subsamples of 0.5 to 1 kg to be used in the laboratory. For this, riffle samplers and sample splitters may be used. The subsamples can be kept in glass or plastic containers. The latter have the advantage that they are unbreakable. Both have the disadvantage is that fine particles may be electrostatically attracted to the container walls thus causing segregation. The rule about labelling is that it should preferably be done on both the container and the lid. If only one label is used this should always be stuck on the container and not on the lid!

3.1.2 Soil Sampling Methods

Properly collecting soil samples is the most important step in any nutrient/soil amendment management program. When considering Soil sampling methods, the following borne in mind:

i. Soil sampling should reflect tillage,
ii. past fertilizer/soil amendment placement,
iii. cropping patterns (and corresponding irrigation requirements),
iv. soil type (including drainage and slope characteristics) and perhaps
v. old field boundaries (such as old feedlots, windrows, altered stream beds, etc.).

Trends toward reduced and/or zero tillage and technology for variable rate fertilization (VRF) have especially demanded that soil samples be taken more comprehensively and intensively for more accurate fertilizer and soil amendment application. This section will discuss the many methods used for taking an accurate soil sample using various methods and under several different types of tillage situations. The most commonly used method for soil sampling would be based on soil types. Fields are split into sampling areas that contain similar soils. Hillsides are kept separate from bottoms since the soil types will vary. Soil survey maps, if applicable, can help organize the soil types throughout the sampling area. Samples will not necessarily need to be collected for every soil type; however, similar soils should be kept together. Sampling maps can be kept to note the locations of the cores for subsequent sampling. The sampling area will be dependent on the soils and topography. Generally, an area of forty acres is considered the maximum size. Smaller sampling areas may be needed if the soils are quite variable or a production problem is apparent.

Once the sampling area is determined, a sufficient number of cores should be taken to acquire a representative sample. This is generally 10 to 20 cores. The depth of sample for surface soils would be 0 to 6 inches or as deep as the primary tillage. Deeper samples to 24 or 36 inches can be taken

for residual nitrate-nitrogen. These deep samples would be kept separate from the surface samples and noted accordingly on the bag and submittal form.

3.1.3 Sampling Techniques

Soil variability is a major concern when trying to obtain a representative soil sample. The strategy used to sample a field can address this challenge. Information collected during a site assessment can assist in choosing an appropriate strategy for a particular field. Some of the sampling techniques that can be adopted include:

- Random composite sampling
- Directed random composite sampling
- Benchmark sampling
- Landscape-directed benchmark sampling
- Grid sampling
- Transect sampling

1. **Random composite sampling**
   Random composite sampling involves taking samples in a random pattern across a field, while avoiding unusual or problem soil areas. This strategy is most appropriate for fields less than 30 ha (80 ac), that have been uniformly cropped in the recent past and have little natural variation. This is the most common method of sampling presently used in most research works in Nigeria. For random sampling, collect cores from 15 to 20 sites and separate each core by depth to obtain representative bulk samples for each depth.
2. **Directed random sampling**
   Directed or managed random sampling is a modified version of a random sampling strategy. This pattern is suited to fields or areas where it is difficult to identify a single dominant area that would represent most of the field. The study field is therefore sub-divided into management zones based on unique characteristics. For instance, if there are noticeable differences in yield throughout a field, management zones might be comprised of below average, average and above-average yielding areas. Take 15 to 20 cores randomly from each management zone. A single field may require several bulk samples depending on the number of management zones. This technique might also be appropriate for areas with more than one soil type, fields with hummocky (rolling) landforms, and fields under strip-crop management.

3. Benchmark sampling

Benchmark sampling involves selecting a small (30 m by 30 m) representative site on a field. This site is used as a guide for fertilizing that entire field. Select probe sampling sites in a grid pattern within the benchmark area and prepare a composite sample for each soil depth. Sampling from the same small area each year reduces sampling variability and better reflects changes in soil nutrient level from year to year. Benchmark sampling sites should be marked with a GPS or by other means. When first using this sampling technique, it can be difficult to select a benchmark site that best represents a field. Therefore, in the first year, it may be necessary to sample and analyze a number of potential benchmark sites. Initially, the costs for laboratory analysis will be higher but the most representative benchmarking site will be identified. If a single site does not adequately represent a field, it may be necessary to maintain multiple benchmark areas (i.e., directed benchmark sampling, see next section).

Features that helps in selecting a benchmark site

- Look for features such as soil colour and landscape to identify where different soil types occur.
- Select a site that has characteristics similar to most of the field or the dominant soil type.
- Observe crop development patterns to assist in identifying different soil conditions. At the beginning of the growing season differences in crop establishment and vigor are more apparent, making a representative location easier to identify.
- Potential benchmark sites can also be selected based on yield,
- Aerial photos or topographic maps, this gaining popularity particularly with increased use of GPS.
- GPS coordinates help to identify and locate the benchmark site for sampling each year.

4. Directed benchmark sampling

Directed benchmark sampling is a variation on the benchmark technique. It involves establishing multiple benchmark areas and management zones, based on topography or other characteristics. This strategy can be used when major areas within fields have distinct and well-defined features related to moisture (e.g., texture, slope). Management zones can be identified using soil surveys, detailed elevation mapping, aerial black and white photographs, yield maps or remote sensed images.

Directed benchmark sampling is only warranted if distinct areas are managed individually. For example, a soil analysis from a benchmark site in a low area suggests that it might respond to higher rates of N compared to a benchmark site on an upland area. Even without variable rate application capabilities, N application could be increased by other means to optimize yield in low areas.

5. **Grid sampling**

Grid sampling is the most intense and expensive sampling strategy. It uses a systematic method to reveal fertility patterns and assumes there is no topographic reason for fertility patterns to vary within a field. For grid sampling, a field is divided into small areas or blocks. A sample location within each block (e.g., the center point) is sampled 3 to 10 times. Sampling frequency may range from one sample from each 60 m × 60 m (0.5 ac) area of the field to one sample from each 2 ha (5 ac) of the field. In general, the smaller the sampling unit, the greater the accuracy.

6. **Transect sampling**

A transect is a path along which one counts and records occurrences of the species of study. It requires an observer to move along a fixed path and to count occurrences along the path and, at the same time (in some procedures), obtain the distance of the object from the path. This results in an estimate of the area covered and an estimate of the way in which detectability increases from probability 0 (far from the path) towards 1 (near the path). Using the raw count and this probability function, one can arrive at an estimate of the actual density of objects.

In soil sampling especially along a slope or a toposequence, a transect is usually made from the crest of the slope to its bottom and sampling carried out along the transect line.

3.1.4 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.

3.1.5 General Soil Sampling Guidelines

- For any soil sampling Technique: Take 15 to 20 cores for each representative bulk sample. This number of samples is based on statistical precision.
- Each core will be segmented into lengths that represent depths of 0 to 15 cm, 15 to 30 cm and 30 to 60 cm.
- Separate the segmented cores by depth into clean, labeled plastic pails. Thoroughly mix the content of each pail, crushing any lumps in the process.
- Avoid using metal pails to collect samples because they can alter the results of micronutrient tests.
- Take a single sub-sample (0.5 kg) for each sampling depth and submit for analysis.
- For hilly fields with knolls, slopes, or depressions, take samples from crest or top slope, mid-slope and foot slope positions to get a representative sample each part of the field.
- Avoid sampling obvious areas of unusual variability such as: saline areas, eroded soils, old manure piles, burn piles, haystacks, corrals, fence rows, old farmsteads or any other unusual areas.
- Soils within 15 m (50 ft) of field borders or shelterbelts and within 50 m (150 ft) of built-up roads should be avoided or sampled separately.
- Always sample prior to manure or fertilizer applications.

3.1.6 Soil Sampling Procedure

1. Prepare a map of the area to be covered in a survey showing different sampling unit boundaries.
2. 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.).
3. 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.
4. Generally, depending on the size of the field, 10–20 spots must be taken for one composite sample.
5. Scrape away surface litter to obtain a uniformly thick slice of soil from the surface to the plough depth from each spot.
6. 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.

7. In this way, collect samples from all the spots marked for one sampling unit.
8. In the case of hard soil, take samples with the help of an auger from the plough depth and collect them in the bucket.
9. 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.
10. 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.
11. The bag used for sampling must always be clean and free from any contamination.

3.1.7 Methods for Sub-Sampling Soils

1. Mixing

The bulk soil sample should be thoroughly homogenized by mixing with a spatula, stirring rod, or other implement. As much of the sample as possible should be loosened and mixed together. No segregation of the sample by aggregate size should be apparent after mixing. Dip into the center of the mixed sample to obtain a subsample.

2. Weighing

Subsamples should be weighed into a tared vessel with a minimum precision of +/- 1% (e.g., 5.0 +/- 0.05 g). If a separate container is used to weigh and transfer subsamples to extraction vessels, it should be brushed out between samples to avoid cross-contamination.

3. Scooping

Dip into the center of the homogenized bulk sample with a standard soil scoop, filling it heaping full without pushing against the side of the soil container. Holding the scoop firmly, tap the handle three times with a spatula, two or three inches from the sample. Hold the spatula perpendicular to the top of the scoop and strike off the excess soil (Peck, 1988). If weight basis results are needed, empty the scoop contents into a tared weighing vessel and record the weight.

3.1.8 Depth of Soil Samples

Remove organic debris, rocks and trash from the surface of the soil sampling areas before the sample is collected the soil sample to plough layer. Select the sampling spot using any of the sampling techniques

discussed earlier. Collect all the samples in a bucker. Depth of sampling is desired according to the purpose.

1. Soil fertility (15-25 cm)
2. Salinity and alkalinity (1m)
3. Establishment of gardens (2m)
4. Soil survey profile of (1-1.5m) depth

Keep record of the areas sampled and simple sketch map for reference.
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.

3.1.9 Steps Adopted In Preparing Samples for Laboratory Analysis

After complete collection of soil samples from field the organic residues like tree leaves, twigs, dung etc. and gravels, stones and other unwanted material should be kept out and sample should be prepared for laboratory analysis by adopting following steps.

1. Drying
2. Grinding
3. Sieving
4. Mixing
5. Partitioning
6. Weighing

7. Labelling
8. Storing

The soil samples should be air dried first and then grinding should be followed. A wooden mortar and pestle should be used for grinding to avoid contamination in the soil sample. After grinding soil samples should be sieved with 2 mm mesh sieve and all the samples are then mixed thoroughly by spreading over cloth or paper. From this bulk soil sample one representative soil sample should be collected following quartering method of portioning. About 250 to 500 g of soil sample is sufficient which should be stored in dry and clean poly bags, screw cap jars or card board boxes with proper labeling.

3.1.10 Sampling Tools and Sample Preparation

A sampling tool should be:

- uncontaminated
- approximately uniform in cross section to the desired depth
- provide reproducible sampling units

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

1. blades: trowel, spade, shovel, spoon, knife, cutlass
2. tubes: open-sided and plain cylinders
3. augers: wood-bit, post-hole, sheathed auger
4. a clean bucket or a tray or a clean cloth – for mixing the soil and subsampling;
5. cloth bags of a specific size;
6. a copying pencil for markings, and tags for tying cloth bags;
7. soil sample information sheet.

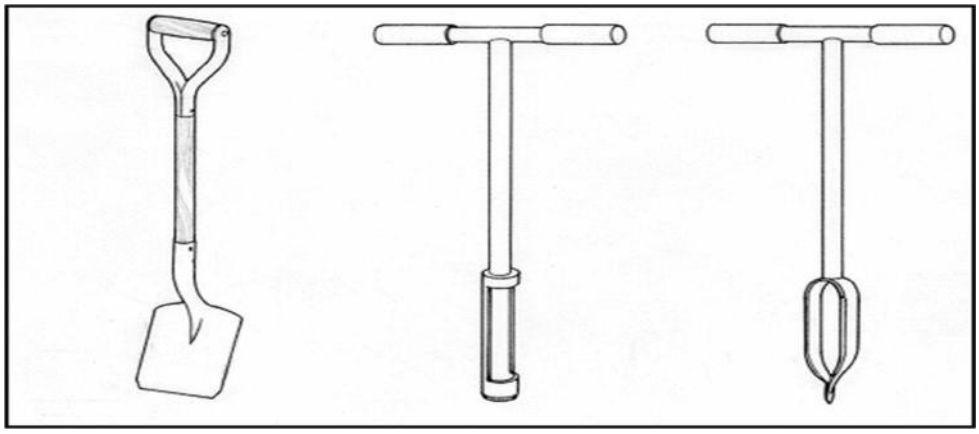

Figure 2. Blade, tube, and auger (left to right).

For comparison over periods of time, take soil samples at approximately the same time of the year (e.g. before planting). When making comparisons, consider other factors like weather conditions, crops, treatments, seasonal fluctuations. Select sampling depth according to the purpose of sampling. For soil fertility evaluations in annual crops, sample at a depth of 0 to 15 or 0 to 20 cm. For perennial crops (e.g. trees), take deeper samples since tree roots often grow deep into the soil.

Soil samples usually need preparation before laboratory analysis:

- **Air-drying.** Crush large soil clods to facilitate drying. Do not dry at high temperature. During air-drying, avoid contamination (i.e. from dust, gases, rain, etc.). Air-drying usually takes one week.
- **Crushing.** Crush the sample in a mortar using a rubber or porcelain-capped pestle (Figure 6). The mortar is usually made from porcelain.

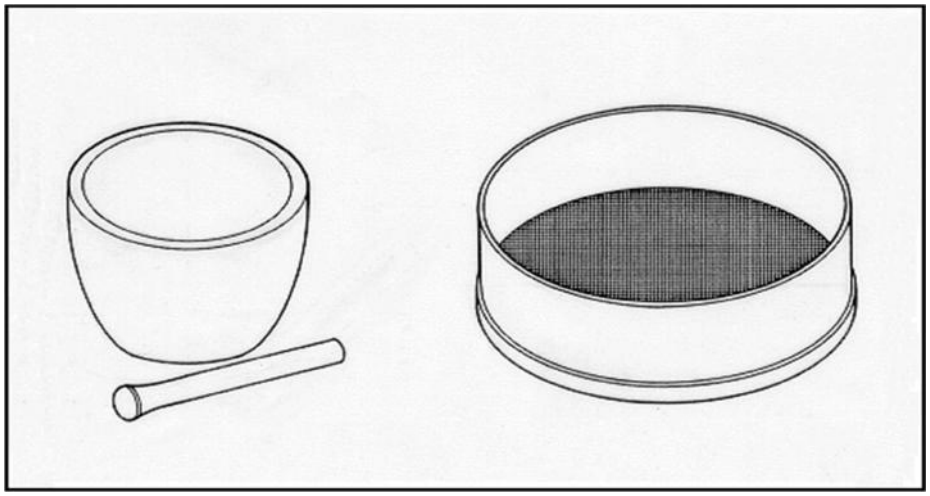

Figure 3. Mortar and pestle, sieve.

- **Pulverising.** Not all samples require pulverising. The subsampling error is a function of the ratio between the average weight of the largest particles and the weight of the subsample. If the subsample being analyzed is small (i.e. for total nitrogen and organic carbon analysis), the sample has to be pulverised to a fine powder (less than 0.5 mm).
- **Sieving.** Sieve the soil through a 2 mm sieve made of brass, stainless steel, or plastic. Use plastic sieves when micronutrients are to be analysed.
- **Mixing and storage.** Mix samples thoroughly, then store in clean closed containers (i.e. polyethylene bags or bottles). Label the containers.

4.0 CONCLUSION

Soil Sampling should be carried out in such a manner that can maximize their use as a soil fertility index based on comparison between sampling events. Consistency, in the areas of season, location (aided by GPS techniques), crop rotation, soil type and sampling depth must be maintained as this will help in proper soil test interpretation. Inconsistencies in any of these areas of soil sampling collection will lessen the interpretation value of soil test changes that occurred since the last soil sample was taken. Along with consistency, soil samples should reflect past soil and fertilizer/amendment management of a given field, taking into account tillage, crop rotation, fertilizer/ amendment placement and also soil characteristics (texture, slope and drainage). Following these guidelines will allow soil tests to be used more effectively for nutrient management and crop diagnostics.

5.0 SUMMARY

You have learnt that the major objectives of soil sampling have been to determine the average nutrient status of a field and to provide some measure of nutrient variability in a field. Through soil sampling and analysis, soil scientists are able to relate trends in soil fertilizer levels to other field properties that are predictable or easily measured. Knowledge of factors influencing soil nutrient levels including soil type, topography, cropping history, manure application, fertilizer application and leveling for irrigation will help the producer determine the most effective sampling approach or technique.

6.0 TUTOR-MARKED ASSIGNMENT

1. What do you understand by soil sampling?
2. What are factors to consider in choosing sampling method?
3. Mention and discuss the sampling techniques you studied.
4. Outline the guidelines for soil sampling.
5. State and explain sampling tools and how they are used for sampling soils.
6. How do we carry out soil preparations before analysis?

7.0 REFERENCES/FURTHER READING

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MODULE 3 USE OF INSTRUMENTS, EQUIPMENTS AND MACHINES

UNIT 1 MICROSCOPY

CONTENTS

1.0 INTRODUCTION

Microscopy comprises of the tools that are used to see/image the microscopic objects and even macromolecules. There exists a wide variety of microscopic tools for studying the bio-molecules and biological processes. Light microscopy is the simplest form of microscopy. This is because it includes all forms of microscopic methods that use electromagnetic radiation to achieve magnification. With the advent of high-resolution microscopes modern microbiologists have access to microscope that produces images with high clarity and magnification. The Leeuwenhoek's singlelens microscope has been transformed in to a high-resolution multi-lens combination with magnification up to two thousand time. Electron microscopes with magnification up too many thousand times and used to study fine structure of cells and sub-cellular components.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- know what microscopy is all about
- understand how microscopes can be used in agricultural laboratories.

3.0 MAIN CONTENT

3.1 Microscopy

The microscope is one of the most important tools of the microbiologist. It was invented in the 1600s when Anton van Leeuwenhoek built on a simple model of a tube, magnifying lens, and stage to make the first visual discoveries of bacteria and circulating blood cells. Nowadays, microscopy is essential in the medical field to make new cellular discoveries, and the types of microscopes can be classified based on the physical principles they use to generate an image. The use of microscopes is crucial in agricultural laboratories especially when analyzing for microbial presence or activities in a plant or animal material. Depending on the type of micro-organism and the purpose of study, microscopes of different capacities are used. Light microscopy uses glass for bending and focusing the light. Refraction (bending) of light is the manifestation of different light velocities in different materials. Refractive index of a material is therefore a measure of the velocity of light in that material. The bending caused in the light beam when it enters from one material into another is given by the Snell's law (fig. 4).

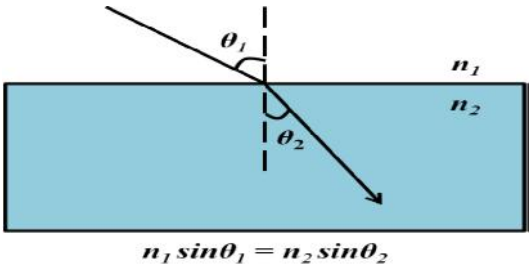

Fig. 4: Snell's law

A convex lens is the simplest microscope. Fig. 3shows how a convex lens produces a magnified image of an object. A light ray parallel to the optical axis of the lens passes through the focus of the lens while a ray passing through the centre of the lens does not bend.

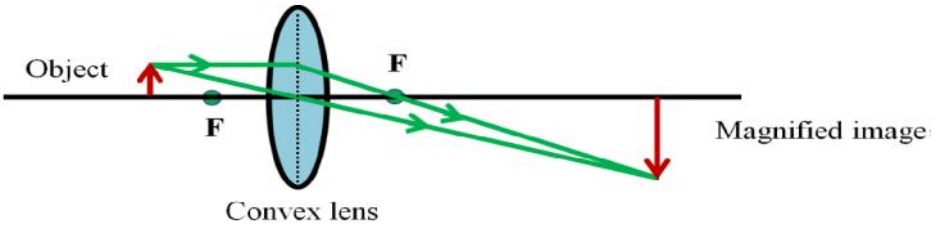

Fig. 5: Magnification of an object by a convex lens

A microscope that uses two lenses to generate the magnified image of the object is called a compound microscope. The magnified image generated by one lens is further magnified by the second lens (Fig. 4). Magnification of a compound microscope is the product of the magnification caused by the objective and ocular (eyepiece) lenses:

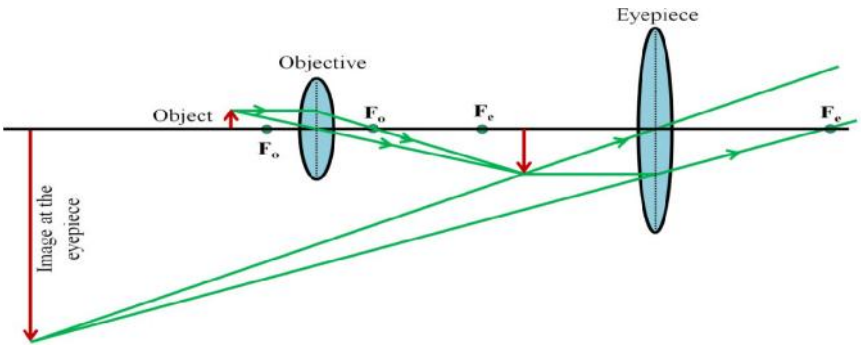

Fig. 6: Ray optical diagram of a compound microscope

3.1.1 Types of Microscope

While most people picture the compound model from lab class when they think of microscopes, many types of microscopes are actually available. These useful devices are employed by researchers, medical technicians and students on a daily basis; the type they select depends on their resources and needs. Some microscopes provide greater resolution with lower magnification and vice versa, and they range in cost from tens to thousands of dollars. There are two major categories of microscopy namely; light (optical) microscopes and electron microscopes.

Microscopy categories

1. Light(optical)microscope and
2. Electronmicroscope

Light microscopy involves use of optical lenses and light radiations and includes

i. Simple microscope
ii. Compound microscope
iii. Stereo Microscope
iv. Confocal microscope
v. Phase-contrast microscope
vi. Dark-field microscope

Electron microcopy is of two types:

i. Transmission microscope and
ii. Scanning Electron microscope

Simple microscope
The simple microscope is generally considered to be the first microscope. It was created in the 17th century by Antony van Leeuwenhoek, who combined a convex lens with a holder for specimens. Magnifying between 200 and 300 times, it was essentially a magnifying glass. While this microscope was simple, it was still powerful enough to provide van Leeuwenhoek information about biological specimens, including the difference in shapes between red blood cells. Today, simple microscopes are not used often because the introduction of a second lens led to the more powerful compound microscope.

Compound microscope
With two lenses, the compound microscope offers better magnification than a simple microscope; the second lens magnifies the image of the first. Compound microscopes are bright field microscopes, meaning that the specimen is lit from underneath, and they can be binocular or monocular. These devices provide a magnification of 1,000 times, which is considered to be high, although the resolution is low. This high magnification, however, allows users to take a close look at objects too small to be seen with the naked eye, including individual cells. Specimens are usually small and have some degree of transparency. Because compound microscopes are relatively inexpensive yet useful, they are used everywhere from research labs to high school biology classrooms

Stereo microscope
The stereo microscope, also called a dissecting microscope, provides magnification of up to 300 times. These binocular microscopes are used to look at opaque objects or objects that are too large to be viewed with a

compound microscope, since they do not require a slide preparation. Although their magnification is relatively low, they are still useful. They provide a close-up, 3-D view of objects' surface textures, and they allow the operator to manipulate the object during viewing. Stereo microscopes are used in biological and medical science applications as well as in the electronics industry, such as by those who make circuit boards or watches.

Confocal microscope
Unlike stereo and compound microscopes, which use regular light for image formation, the confocal microscope uses a laser light to scan samples that have been dyed. These samples are prepared on slides and inserted; then, with the aid of a dichromatic mirror, the device produces a magnified image on a computer screen. Operators can create 3-D images, as well, by assembling multiple scans. Like the compound microscope, these microscopes offer a high degree of magnification, but their resolution is much better. They are commonly used in cell biology and medical applications.

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 orphase 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.

The electron microscope

The energy source used in the electron microscope is a beam of electrons. The beam has an exceptionally short wavelength, and increases the resolution of the image significantly over light microscopy. Whole objects are coated in gold or palladium, which deflects the electron beam, creating dark and light areas as 3-D images viewed on a monitor. Details like the intricate silica shells of marine diatoms and surface details of viruses can be captured. Both transmission electron microscopes (TEM) and the newer scanning electron microscopes (SEM) fall in this specialized category of microscopy.

Transmission Electron Microscope (TEM)

Like the scanning electron microscope, the transmission electron microscope (TEM) uses electrons in creating a magnified image, and samples are scanned in a vacuum so they must be specially prepared. Unlike the SEM, however, the TEM uses a slide preparation to obtain a 2-D view of specimens, so it's more suited for viewing objects with some degree of transparency. A TEM offers a high degree of both magnification and resolution, making it useful in the physical and biological sciences, metallurgy, nanotechnology and forensic analysis.

Scanning Electron Microscope (SEM)

The scanning electron microscope, or SEM, uses electrons rather than light for image formation. Samples are scanned in vacuum or near-vacuum conditions, so they must be specially prepared by first undergoing dehydration and then being coated with a thin layer of a conducive material, such as gold. After the item is prepared and placed in the chamber, the SEM produces a 3-D, black-and-white image on a computer screen. Offering ample control over the amount of magnification, SEMs are used by researchers in the physical, medical and biological sciences to examine a range of specimens from insects to bones.

Objectives of microscopes

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.

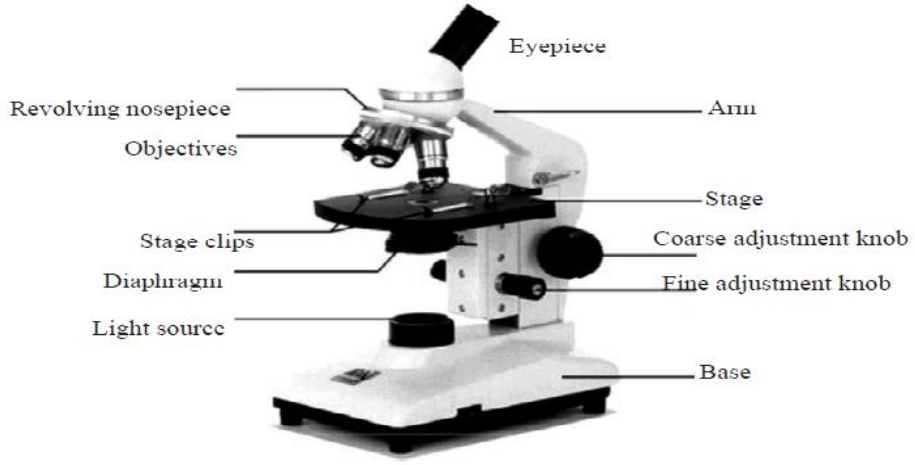

Fig. 7: Parts of optical microscope

Functions of parts of optical microscope

i. **Light source**: project aparallel beam of light on to the sample for illumination
ii. **Condenser** gathers light and concentrates it into a cone of light that illuminates the specimen with uniform intensity over the entire view field
iii. **Sample stage:** with X-Y movement: sample is placed on the stage and different part of the sample can be viewed due to the X-Y movement capability
iv. **Fine/Focusing knobs**: since the distance between objective and eye piece is fixed, focusing is achieved by moving the sample relative to the objective lens.
v. **Objectives**: does the main part of magnification and resolves the fine details on the samples (*m*o~10–100)
vi. **Eyepiece**: forms a further magnified virtual image which can be observed directly with eyes (*m*e~10)
vii. **Beam splitter and camera**: allow a permanent record of the real image from the objective be made on film (for modern research microscope).

The procedure for using a microscope is:

i. 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.
ii. Place the slide containing the specimen on the stage.

iii. Keeping the eye close to the eyepiece, turn the coarse adjustment slowly to raise the body tube until the slide comes into focus.
iv. Turn the fine adjustment slowly until the focus is perfect and the object being examined is seen clearly.
v. 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.
vi. 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.
vii. Then study/examine the specimen.

4.0 CONCLUSION

The main types of microscopes used in Agricultural laboratories study are simple and 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.

5.0 SUMMARY

While most people picture the compound model from lab class when they think of microscopes, many types of microscopes are actually available. These useful devices are employed by researchers, medical technicians and students on a daily basis; the type they select depends on their resources and needs. Some microscopes provide greater resolution with lower magnification and vice versa, and they range in cost from tens to thousands of dollars. There are two major categories of microscopy namely; light (optical) microscopes and electron microscopes.

6.0 TUTOR-MARKED ASSIGNMENT

1. Briefly explain the term “microscopy” and state its use.
2. Give brief historical background of the development of microscopy.
3. State the two (2) major categories of microscopes and give the different types under each.
4. With the aid of a labelled diagram, show the parts of an optic microscope.
5. What are the functions of different parts of an optic microscope?
6. Outline the procedure involved in using a microscope.

7.0 REFERENCES/FURTHER READING

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UNIT 2 PRINCIPLES AND OPERATION OF ATOMIC ABSORPTION SPECTROPHOTOMETER (AAS)

CONTENTS

1.0 INTRODUCTION

Trace element analysis is very important in many fields which encompass ecological science, food analysis, forensic science, material science, soil and plant science as well as in environmental studies. In order to carry out the analysis, many techniques varying in degree of sensitivity and convenience have been developed. These include Atomic Absorption Spectrometry, Inductively Coupled Plasma-atomic emission Spectrometry, X-ray Fluorescence, Neutron Activation analysis, Mass Spectrometry and Proton Induced x-ray Emission. Atomic Absorption Spectrometry will be discussed in this unit. It is worthy to note that Atomic Absorption Spectrometer and Atomic Absorption Spectrophotometer are always used interchangeably and both are always abbreviated as AAS, a few slight differences have been noted; a spectrometer tells you which wavelengths of light is absorbed and which wavelengths of light is reflected. A spectrophotometer measures the relative intensity of the light absorbed or reflected at a particular wavelength of light. Spectrometer is a term that applied to instruments that operate over a very wide range of wavelengths, from gamma rays and x-rays into the far infrared, however if the instrument is designed to measure the spectrum in absolute units rather than relative units, then it is typically called spectrophotometer. In this unit, we will use the two interchangeably.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- understand the principles and operations of AAS

- know the different components of the AAS and functions

3.0 MAIN CONTENT

3.1 Atomic Absorption Spectrophotometer (AAS)

Atomic absorption spectrometry (AAS) is an analytical technique that measures the concentrations of elements. Atomic absorption is so sensitive that it can measure down to parts per billion of a gram (μgdm–3) in a sample. The technique makes use of the wavelengths of light specifically absorbed by an element. They correspond to the energies needed to promote electrons from one energy level to another, higher, energy level. Atomic absorption spectrometry has many uses in different areas of chemistry.

Fig. 8: Flame atomic absorption spectroscopy instrument (Wikipedia)

3.1.1 How it Works

- Atoms of different elements absorb characteristic wavelengths of light.
- Analysing a sample to see if it contains a particular element means using light from that element.
- For example with lead, a lamp containing lead emits light from excited lead atoms that produce the right mix of wavelengths to be absorbed by any lead atoms from the sample.
- In AAS, the sample is atomised – *ie* converted into ground state free atoms in the vapour state – and a beam of electromagnetic radiation emitted from excited lead atoms is passed through the vaporised sample.
- Some of the radiation is absorbed by the lead atoms in the sample. The greater the number of atoms there is in the vapour, the more

radiation is absorbed. The amount of light absorbed is proportional to the number of lead atoms.

- A calibration curve is constructed by running several samples of known lead concentration under the same conditions as the unknown. The amount the standard absorbs is compared with the calibration curve and this enables the calculation of the lead concentration in the unknown sample.
- Consequently, an atomic absorption spectrometer needs the following three components: a light source; a sample cell to produce gaseous atoms; and a means of measuring the specific light absorbed.

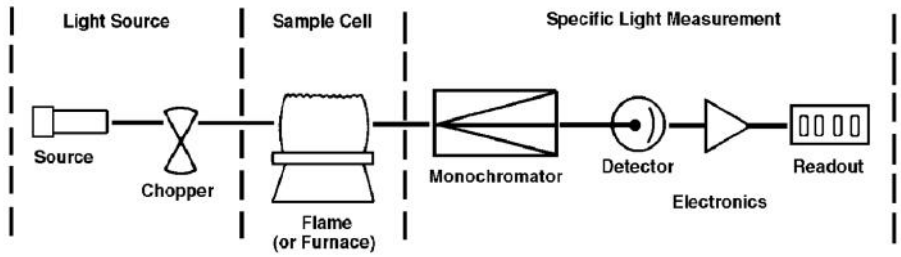

Fig. 9: Basic Atomic Absorption Spectrometer

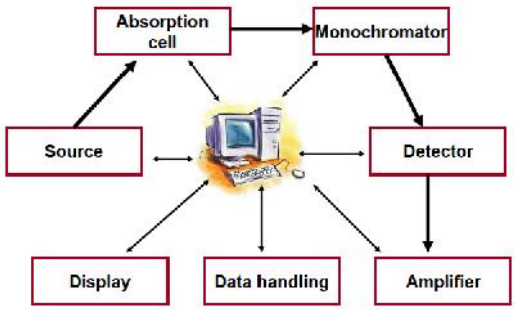

Fig.10: Schematic Diagram of Atomic Absorption Spectrometer

3.1.3 Principles of Operation of AAS

The technique makes use of the atomic absorption spectrum of a sample in order to assess the concentration of specific analytes within it. It requires standards with known analyte content to establish the relation between the measured absorbance and the analyte concentration and relies therefore on the Beer-Lambert Law.

In short, the electrons of the atoms in the atomizer can be promoted to higher orbitals (excited state) for a short period of time (nanoseconds) by absorbing a defined quantity of energy (radiation of a given wavelength). This amount of energy, i.e., wavelength, is specific to a particular electron transition in a particular element. In general, each wavelength corresponds to only one element, and the width of an absorption line is only of the order of a few picometers (pm), which gives the technique its elemental selectivity. The radiation flux without a sample and with a sample in the atomizer is measured using a detector, and the ratio between the two values (the absorbance) is converted to analyte concentration or mass using the Beer-Lambert Law.

3.1.4 The Basic Components

Every absorption spectrometer must have components which fulfill the three basic requirements. There must be:

1. **Light source:** A light source which emits the sharp atomic lines of the element to be determined is needed. The most widely known and recognised source is the hollow cathode lamp. These lamps are designed to emit the atomic spectrum of a particular element, and specific lamps are selected for use depending on the element to be determined.
2. **Sample cell:** An atomic vapor must be generated in the light beam from the source. This is generally accomplished by introducing the sample into a burner system or electrically heated furnace aligned in the optical path of the spectrophotometer.
3. **Means of specific light measurement:** The selection of a specific source and a particular wavelength in that source is what allows the determination of a selected element to be made in the presence of others. The wavelength of light which is isolated by the monochromator is directed onto the detector, which serves as the ‘‘eye’’ of the instrument.

1. Light sources

An atom absorbs light at discrete wavelengths. In order to measure this narrow light absorption with maximum sensitivity, it is necessary to use a line source, which emits the specific wavelengths which can be absorbed by the atom. Narrow line sources not only provide high sensitivity, but also make atomic absorption a very specific analytical technique with few spectral interferences. The two most common line sources used in atomic absorption are the ‘‘hollow cathode lamp’’ and the ‘‘electrodeless discharge lamp.’’

i. The hollow cathode lamp

The hollow cathode lamp is an excellent, bright line source for most of the elements determinable by atomic absorption.

ii. The electrodeless discharge lamp

The atomic absorption determination of these elements can often be dramatically improved with the use of brighter, more stable sources such as the ‘‘electrodeless discharge lamp’’.

2. Optical considerations

Photometers

The portion of an atomic absorption spectrometer’s optical system which conveys the light from the source to the monochromator is referred to as the *photometer*. Three types of photometers are typically used in atomic absorption instruments: single-beam, double-beam and what might be called compensated single-beam or pseudo double-beam.

i. Single-beam photometers

The instrument represents a fully functional ‘‘single-beam’’ atomic absorption spectrometer. It is called ‘‘single-beam’’ because all measurements are based on the varying intensity of a single beam of light in a single optical path.

ii. Double-beam photometers

An alternate photometer configuration, known as ‘‘double-beam’’ uses additional optics to divide the light from the lamp into a ‘‘sample beam’’ (directed through the sample cell) and a ‘‘reference beam’’ (directed around the sample cell).

iii. Alternative photometer designs

Many alternative system designs which provide advantages that are similar to those of double beam optical systems and the light throughput characteristic of single-beam systems.

3. The atomic absorption atomiser (The Sample Cell)

i. Pre-mix burner system

The sample cell, or atomiser, of the spectrometer must produce the ground state atoms necessary for atomic absorption to occur. This involves the application of thermal energy to break the bonds that hold atoms together as molecules.

ii. Impact devices

The sample aerosol is composed of variously sized droplets as it is sprayed into the mixing chamber. Upon entering the flame, the water in these droplets is vaporised. The remaining solid material must likewise be vaporised, and chemical bonds must be broken to create free ground state atoms.

iii. Nebulisers, burner heads and mounting systems

- Several important factors enter into the nebulizer portion of the burner system. In order to provide efficient nebulization for all types of sample solution, the nebulizer should be adjustable. Stainless steel has been the most common material used for construction of the nebuliser because of its durability and affordability.
- Burner heads typically are constructed of stainless steel or titanium. All-titanium heads are preferred as they provide extreme resistance to heat and corrosion.
- A ‘‘quick change’’ atomiser mount is an important item to facilitate convenient changeover from one device to another without the use of tools.

4. Electronics

i. Precision in atomic absorption measurements

Observed precision will improve with the period of time over which each sample is read. Where analyte concentrations are not approaching detection limits, integration times of one to three seconds will usually provide acceptable precision. When approaching instrument detection limits where repeatability is poor, precision can be improved by using even longer integration times, up to 10 seconds. In most instances; however, there is little advantage to using integration times longer than 10 seconds.

ii. Calibration of the spectrometer

- Most modern atomic absorption instruments include microcomputer-based electronics.
- The microcomputer provides atomic absorption instruments with advanced calculation capabilities, including the ability to calibrate and compute concentrations from absorbance data conveniently and accurately, even for nonlinear calibration curves.
- In the linear region, data on as little as one standard and a blank may be sufficient for defining the relationship between concentration and absorbance.

- However, additional standards are usually used to verify calibration accuracy. Where the relationship becomes nonlinear, however, more standards are required.
- The accuracy of a calibration computed for a nonlinear relationship depends on the number of standards and the equations used for calibration.
- For the equation format which optimally fits atomic absorption data, it has been experimentally shown that accurate calibration can be achieved with a minimum of three standards plus a blank, even in cases of severe curvature.

    - Figure 11 illustrates the accuracy of microcomputer-calculated results for cobalt with single standard ‘‘linear’’ and three-standard ‘‘nonlinear’’ calibrations. After the instrument was calibrated using the specified procedure, a series of standards were analyzed.
    - Figure 11 shows plots of the actual concentrations for those standards versus the measured values for both calibration procedures.
    - The results obtained with ‘‘linear’’ calibration are accurate only where the absorbance: concentration relationship is linear, up to about 5 mg/mL.
    - The results obtained with three-standard ‘‘nonlinear’’ calibration are still accurate at 30mg/mL, significantly extending the useful working range. For versatility, current instruments allow fitting of more than three standards to these same basic equations.

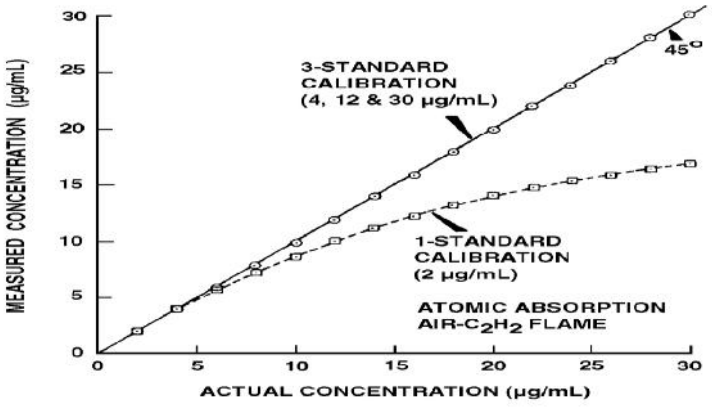

Fig. 11: Cobalt Calibration Accuracy

5. Automation of Atomic Absorption

i. **Automated Instruments and Sample Changers**
One of the greatest contributions to the efficiency of the analytical laboratory is the automated atomic absorption spectrometer. Automatic samplers were the first step in freeing the analyst from the monotonous task of manually introducing each and every sample.

ii. **Automated sample preparation**
The introduction of commercial systems based on techniques such as flow injection have directly addressed the need for automated sample preparation capabilities. Flow injection techniques can be used to automate relatively simple procedures such as dilution or reagent addition. They can also be used to automate complex chemical pretreatments, including analyte preconcentration and cold vapor mercury and hydride generation procedures.

iii. **The stand-alone computer and atomic absorption**
Stand-alone and personal computers have extended the automation and data handling capabilities of atomic absorption even further. These computers can interface directly to instrument output ports to receive, manipulate, and store data and print reports in user selectable formats. Also, data files stored in personal computers can be read into supplemental software supplied with the system or third-party software such as word processor, spreadsheet and database programs for open-ended customization of data treatment and reporting.

4.0 CONCLUSION

You have learnt that atoms of different elements absorb characteristic wavelengths of light. Analysing a sample to see if it contains a particular element means using light from that element. For e.g. with lead, a lamp containing lead emits light from excited lead atoms that produce the right mix of wavelengths to be absorbed by any lead atoms from the sample. In AAS, the sample is atomised – *ie* converted into ground state free atoms in the vapour state – and a beam of electromagnetic radiation emitted from excited lead atoms is passed through the vaporised sample. Some of the radiation is absorbed by the lead atoms in the sample. The greater the number of atoms there is in the vapour, the more radiation is absorbed. The amount of light absorbed is proportional to the number of lead atoms. A calibration curve is constructed by running several samples of known lead concentration under the same conditions as the unknown.

5.0 SUMMARY

You have been taught that the three main requirements that the components of every AAS must fulfill include Light source, Sample cell and means of specific light measurement. A light source which emits the sharp atomic lines of the element to be determined. Sample cell ensures an atomic vapor must be generated in the light beam from the source. This is generally accomplished by introducing the sample into a burner system or electrically heated furnace aligned in the optical path of the spectrophotometer. Means of specific light measurement: The selection of a specific source and a particular wavelength in that source is what allows the determination of a selected element to be made in the presence of others.

6.0 TUTOR-MARKED ASSIGNMENT

1. Briefly explain the AAS instrument and mention 5 other instruments in that category.
2. Explain how the AAS instrument works and state its principles of operation.
3. Mention and briefly explain the three basic components of the AAS.
4. What do you understand as the Photometer? State three types of photometers you studied.
5. Write short notes on the following components of a Sample cell or Atomic Absorption Atomiser.
   i. Pre-Mix Burner System.
   ii. Impact devices.
   iii. Nebulisers, burner heads and mounting systems.
6. Give a detailed explanation on how calibration of the spectrometer can be carried out.
7. What is the difference and similarity of colorimeter to AAS?

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UNIT 3 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

CONTENTS

1.0 INTRODUCTION

High-performance liquid chromatography (**HPLC**; also formerly known as **high-pressure liquid chromatography**) is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. This equipment relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out of the column. HPLC has been used for manufacturing (*e.g.*, during the production process of pharmaceutical and biological products), legal (*e.g.*, detecting performance enhancement drugs in urine), research (*e.g.*, separating the components of a complex biological sample, or of similar synthetic chemicals from each other), and medical (*e.g.*, detecting vitamin D levels in blood serum) purposes.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- know what HPLC is all about
- understand how HPLC operates in agricultural and microbiological laboratories.

3.0 MAIN CONTENT

3.1 High-Performance Liquid Chromatography (HPLC)

HPLC is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. Chromatography can be described as a mass transfer process involving adsorption. Chromatography – is an analytical technique whereby a sample is separated into its individual components. During separation the sample components are distributed between a stationary phase and a mobile phase. After separation the components can be quantified and even identified. HPLC relies on pumps to pass a pressurized liquid and a sample mixture through a column filled with adsorbent, leading to the separation of the sample components. The active component of the column, the adsorbent, is typically a granular material made of solid particles (*e.g.*, silica, polymers, etc.), 2–50 μm in size. The components of the sample mixture are separated from each other due to their different degrees of interaction with the adsorbent particles. The pressurized liquid is typically a mixture of solvents (*e.g.*, water, acetonitrile and/or methanol) and is referred to as a "mobile phase". Its composition and temperature play a major role in the separation process by influencing the interactions taking place between sample components and adsorbent.

These interactions are physical in nature, such as hydrophobic (dispersive), dipole–dipole and ionic, most often a combination. The process of operation of a HPLC is as shown in Fig. 9

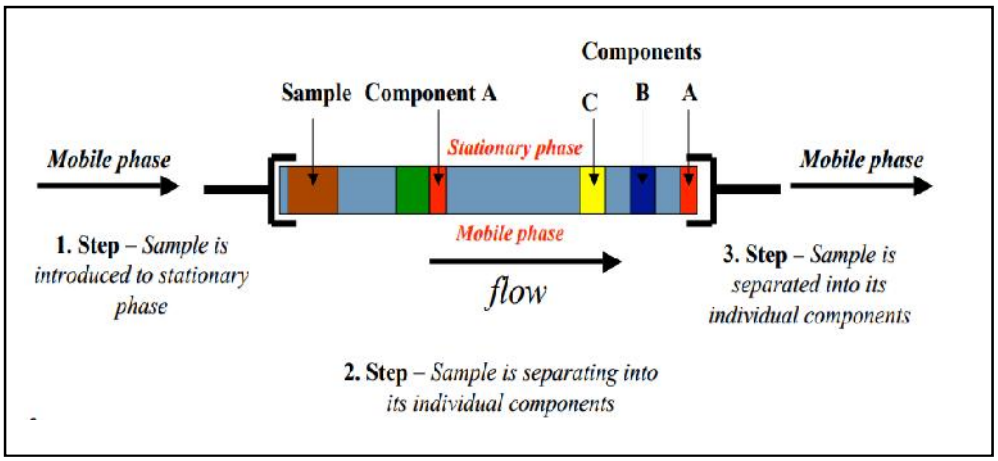

Fig. 12: Process of operation of a HPLC

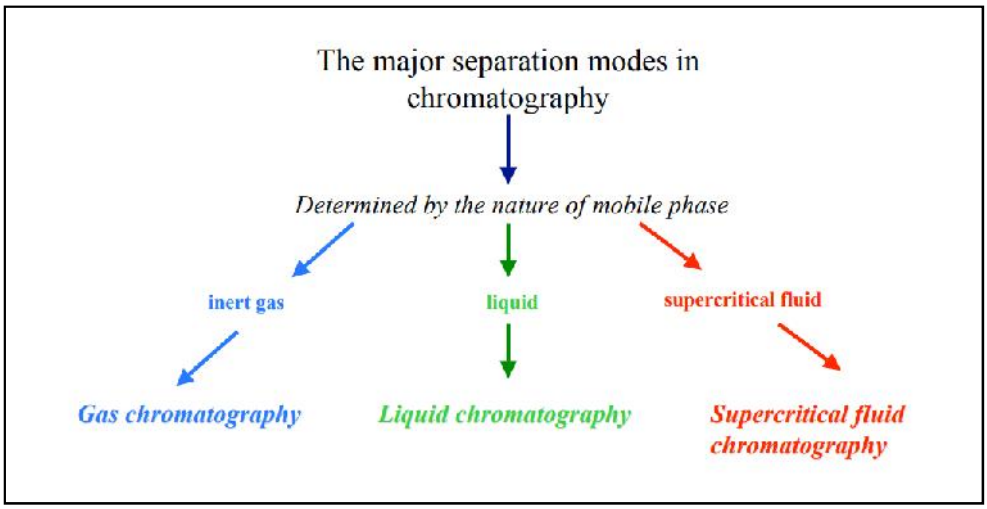

Fig. 13: Separation modes in chromatography

3.1.1 Historical Perspective

- 1903 Tswett - plant pigments separated on chalk columns
- 1931 Lederer& Kuhn - LC of carotenoids
- 1938 TLC and ion exchange
- 1950 reverse phase LC
- 1954 Martin & Synge (Nobel Prize)
- 1959 Gel permeation
- 1965 instrumental LC (Waters)

The term High Performance Liquid Chromatography (HPLC) was introduced in the 1970's to distinguish the modern high-performance technique from classical low-pressure column chromatography, developed in the 1930's.

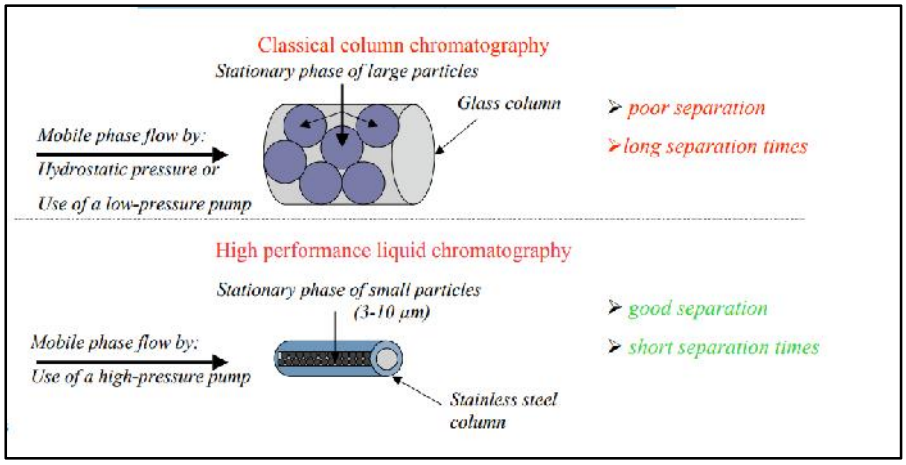

Fig. 14: Difference between classical column chromatography and HPLC

Table 4: Difference between classical column chromatography and HPLC

	Classical Column Chromatography	High Performance Liquid Chromatography (HPLC)
1.	Its mobile phase flow is by Hydrostatic pressure or use of low-pressure pump	Its mobile phase flow is by a high-pressure pump
2	Stationary phase made up of large particles	Stationary phase of small particles (3 - 10µm)
3	Made up of glass column	Stainless steel column
4	Long separation time	Short separation time
5	Poor separation	Good separation

Table 5: Improvement in Separation Efficiency

Year	Particle diameter (µm)	Separation power N/m
Classical LC	100µm	100
1965	30	10,000
1971	10	30,000
1975	5	60,000
1978	3	100,000
1990	1.5	360,000

3.1.2 HPLC is Very Applicable in Separating Substances Such As:

- Amino acids
- Proteins
- Nucleic acids
- Carbohydrates
- Terpenoids
- Antibiotics
- Steroids
- Inorganic salts

3.1.3 Major HPLC Application Areas Are

1. Quality control in industry

2. Determination of active compounds in drug research and the pharmaceutical industry
3. Polymer analysis
4. Determination of toxic compounds in environmental analysis environmental analysis
5. Quality control in the food and cosmetics industry
6. Separation and isolation of biopolymers such as enzymes and nucleic acids
7. Manufacturing: HPLC has many applications in both laboratory and clinical science. It is a common technique used in pharmaceutical development, as it is a dependable way to obtain and ensure product purity.
8. Legal: This technique is also used for detection of illicit drugs in urine. The most common method of drug detection is an immunoassay. This method is much more convenient. However, convenience comes at the cost of specificity and coverage of a wide range of drugs.
9. Research: Similar assays can be performed for research purposes, detecting concentrations of potential clinical candidates like anti-fungal and asthma drugs. This technique is obviously useful in observing multiple species in collected samples, as well, but requires the use of standard solutions when information about species identity is sought out.
10. Medical: Medical use of HPLC always include drug analysis, it however falls more closely under the category of nutrient analysis. While urine is the most common medium for analyzing drug concentrations, blood serum is the sample collected for most medical analyses with HPLC. Other methods of detection of molecules that are useful for clinical studies have been tested against HPLC, namely immunoassays.

3.1.4 The HPLC Parameters

1. **Theoretical:** HPLC separations have theoretical parameters and equations to describe the separation of components into signal peaks when detected by instrumentation such as by a UV detector or a mass spectrometer.
2. **Internal diameter:** The internal diameter (ID) of an HPLC column is an important parameter that influences the detection sensitivity and separation selectivity in gradient elution.
3. **Particle size:** Most traditional HPLC is performed with the stationary phase attached to the outside of small

spherical silica particles (very small beads). These particles come in a variety of sizes with five µm beads being the most common.

4. **Pore size:** Many stationary phases are porous to provide greater surface area. Small pores provide greater surface area while larger pore size has better kinetics, especially for larger analytes.
5. **Pump pressure:** Pumps vary in pressure capacity, but their performance is measured on their ability to yield a consistent and reproducible volumetric flow rate. Pressure may reach as high as 60 MPa (6000 lbf/in^2), or about 600 atmospheres. Modern HPLC systems have been improved to work at much higher pressures, and therefore are able to use much smaller particle sizes in the columns (<2 µm).
6. **Detectors:** HPLC detectors fall into two main categories: universal or selective. Universal detectors typically measure a bulk property (*e.g.*, refractive index) by measuring a difference of a physical property between the mobile phase and mobile phase with solute while selective detectors measure a solute property (*e.g.*, UV-Vis absorbance) by simply responding to the physical or chemical property of the solute.
7. **Autosamplers:** Large numbers of samples can be automatically injected onto an HPLC system, by the use of HPLC autosamplers. In addition, HPLC autosamplers have an injection volume and technique which is exactly the same for each injection, consequently they provide a high degree of injection volume precision.

3.1.5 Principles of HPLC (High Performance Liquid Chromatography)

- HPLC is distinguished from traditional ("low pressure") liquid chromatography because operational pressures are significantly higher (50–350 bar), while ordinary liquid chromatography typically relies on the force of gravity to pass the mobile phase through the column.
- The schematic of a HPLC instrument typically includes a degasser, sampler, pumps, and a detector. The sampler brings the sample mixture into the mobile phase stream which carries it into the column.
- The pumps deliver the desired flow and composition of the mobile phase through the column.
- The detector generates a signal proportional to the amount of sample component emerging from the column, hence allowing for quantitative analysis of the sample components.

- A digital microprocessor and user software control the HPLC instrument and provide data analysis.
- Some models of mechanical pumps in a HPLC instrument can mix multiple solvents together in ratios changing in time, generating a composition gradient in the mobile phase.
- Various detectors are in common use, such as UV/Vis, photodiode array (PDA) or based on mass spectrometry.
- Most HPLC instruments also have a column oven that allows for adjusting the temperature at which the separation is performed.

3.1.6 Types of HPLC

1. Partition chromatography

Partition chromatography was one of the first kinds of chromatography that chemists developed. The partition co efficient principle has been applied in paper chromatography, thin layer chromatography, gas phase and liquid–liquid separation applications.

2. Normal–phase chromatography

Normal–phase chromatography was one of the first kinds of HPLC that chemists developed. Also known as normal-phase HPLC (NP-HPLC) this method separates analytes based on their affinity for a polar stationary surface such as silica, hence it is based on analyte ability to engage in polar interactions (such as hydrogen-bonding or dipole-dipole type of interactions) with the sorbent surface. NP-HPLC uses a non-polar, non-aqueous mobile phase (*e.g.*, Chloroform), and works effectively for separating analytes readily soluble in non-polar solvents.

3. Displacement chromatography

The basic principle of displacement chromatography is: A molecule with a high affinity for the chromatography matrix (the displacer) will compete effectively for binding sites, and thus displace all molecules with lesser affinities. There are distinct differences between displacement and elution chromatography. In elution mode, substances typically emerge from a column in narrow, Gaussian peaks. Wide separation of peaks, preferably to baseline, is desired in order to achieve maximum purification. The speed at which any component of a mixture travels down the column in elution mode depends on many factors. But for two substances to travel at different speeds, and thereby be resolved, there must be substantial differences in some interaction between the biomolecules and the chromatography matrix.

i. Reversed-phase chromatography (RPC)

Reversed phase HPLC (RP-HPLC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is a silica which has been surface-modified with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. With such stationary phases, retention time is longer for molecules which are less polar, while polar molecules elute more readily (early in the analysis). An investigator can increase retention times by adding more water to the mobile phase; thereby making the affinity of the hydrophobic analyte for the hydrophobic stationary phase stronger relative to the now more hydrophilic mobile phase.

ii. Size-exclusion chromatography

Size-exclusion chromatography (SEC), also known as *gel permeation chromatography* or *gel filtration chromatography*, separates particles on the basis of molecular size (actually by a particle's Stokes radius). It is generally a lowresolution chromatography and thus it is often reserved for the final, "polishing" step of the purification. It is also useful for determining the tertiary structure and quaternary structure of purified proteins. SEC is used primarily for the analysis of large molecules such as proteins or polymers. SEC works by trapping these smaller molecules in the pores of a particle. The larger molecules simply pass by the pores as they are too large to enter the pores. Larger molecules therefore flow through the column quicker than smaller molecules, that is, the smaller the molecule, the longer the retention time.

iii. Ion-exchange chromatography

The ion-exchange chromatography (IC) operation is such that retention is based on the attraction between solute ions and charged sites bound to the stationary phase. Solute ions of the same charge as the charged sites on the column are excluded from binding, while solute ions of the opposite charge of the charged sites of the column are retained on the column. Solute ions that are retained on the column can be eluted from the column by changing the solvent conditions (*e.g.*, increasing the ion effect of the solvent system by increasing the salt concentration of the solution, increasing the column temperature, changing the pH of the solvent, etc.).

iv. Bio-affinity chromatography

This chromatographic process relies on the property of biologically active substances to form stable, specific, and reversible complexes. The formation of these complexes involves the participation of common molecular forces such as the Van der Waals interaction, electrostatic interaction, dipole-dipole interaction, hydrophobic

interaction, and the hydrogen bond. An efficient, biospecific bond is formed by a simultaneous and concerted action of several of these forces in the complementary binding sites.

v. Aqueous normal-phase chromatography

Aqueous normal-phase chromatography (ANP) is a chromatographic technique which encompasses the mobile phase region between reversed-phase chromatography (RP) and organic normal phase chromatography (ONP). This technique is used to achieve unique selectivity for hydrophilic compounds, showing normal phase elution using reversed-phase solvents.

4.0 CONCLUSION

HPLC is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. Chromatography can be described as a mass transfer process involving adsorption. Chromatography – is an analytical technique whereby a sample is separated into its individual components. During separation the sample components are distributed between a stationary phase and a mobile phase. After separation the components can be quantified and even identified. Different types of HPLC includes; Partition, Normal phase, Displacement, Reversed phase, Size-exclusion, Ion- exchange, Bio-affinity and Aqueous normal-phase chromatography.

5.0 SUMMARY

The High Performance Liquid Chromatography (HPLC) equipment relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out of the column. HPLC has been used for manufacturing (*e.g.*, during the production process of pharmaceutical and biological products), legal (*e.g.*, detecting performance enhancement drugs in urine), research (*e.g.*, separating the components of a complex biological sample, or of similar synthetic chemicals from each other), and medical (*e.g.*, detecting vitamin D levels in blood serum) purposes.

6.0 TUTOR-MARKED ASSIGNMENT

1. What do you understand as HPLC? Give a brief historical background of its development.
2. In a tabular form give five (5) differences between Classical column chromatography and HPLC.
3. Outline eight (8) substances that can be separated with HPLC and what are other areas of its application.
4. State at least five operational principles of the HPLC equipment.
5. Name and briefly discuss at least five (5) different types of HPLC.
6. Enumerate and briefly explain at least five (5) parameters of HPLC.

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UNIT 4 THIN LAYER CHROMATOGRAPHY (TLC)

CONTENTS

1.0 INTRODUCTION

Thin-layer chromatography (TLC) is a chromatography technique used to separate non-volatile mixtures. Thin-layer chromatography is normally carried out on a sheet of glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide (alumina), or cellulose. This layer of adsorbent is known as the stationary phase. After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved. The mobile phase has different properties from the stationary phase. For example, with silica gel, a very polar substance, non-polar mobile phases such as heptane are used. The mobile phase may be a mixture, allowing chemists to fine-tune the bulk properties of the mobile phase. After the experiment, the spots are visualized. Often this can be done simply by projecting ultraviolet light onto the sheet; the sheets are treated with a phosphor, and dark spots appear on the sheet where compounds absorb the light impinging on a certain area. Chemical processes can also be used to visualize spots; anisaldehyde, for example, forms colored adducts with many compounds, and sulfuric acid will char most organic compounds, leaving a dark spot on the sheet.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- know what thin layer chromatography is

- know how to use the TLC in mixture separation

3.0 MAIN CONTENT

3.1 Thin Layer Chromatography (TLC)

Thin-layer chromatography (TLC) is a chromatography technique used to separate non-volatile mixtures. Thin-layer chromatography can be used to monitor the progress of a reaction, identify compounds present in a given mixture, and determine the purity of a substance. Specific examples of these applications include: analysing ceramides and fatty acids, detection of pesticides or insecticides in food and water, analysing the dye composition of fibers in forensics, as saying the radiochemical purity of radiopharmaceuticals, or identification of medicinal plants and their constituents. A number of enhancements can be made to the original method to automate the different steps, to increase the resolution achieved with TLC and to allow more accurate quantitative analysis. This method is referred to as HPTLC, or "high-performance TLC". HPTLC typically uses thinner layers of stationary phase and smaller sample volumes, thus reducing the loss of resolution due to diffusion.

3.1.1 Preparation of the Plate

TLC plates are usually commercially available, with standard particle size ranges to improve reproducibility. They are prepared by mixing the adsorbent, such as silica gel, with a small amount of inert binder like calcium sulfate (gypsum) and water. This mixture is spread as a thick slurry on an unreactive carrier sheet, usually glass, thick aluminum foil, or plastic. The resultant plate is dried and *activated* by heating in an oven for thirty minutes at 110 °C. The thickness of the absorbent layer is typically around 0.1 – 0.25 mm for analytical purposes and around 0.5 – 2.0 mm for preparative TLC.

3.1.2 Procedure

The process is the same as paper chromatography with the advantage of faster runs, better separations, and the choice between different stationary phases. Because of its simplicity and speed TLC is often used for monitoring chemical reactions and for the qualitative analysis of reaction products. Plates can be labeled before or after the chromatography process using a pencil or other implement that will not interfere or react with the process.

To run a thin layer chromatography plate, the following procedure is carried out:

- Using a capillary, a small spot of solution containing the sample is applied to a plate, about 1.5 centimeters from the bottom edge. The solvent is allowed to completely evaporate off to prevent it from interfering with sample's interactions with the mobile phase in the next step.
- A small amount of an appropriate solvent (eluent) is poured into a glass beaker or any other suitable transparent container (separation chamber) to a depth of less than 1 centimeter. A strip of filter paper (aka "wick") is put into the chamber so that its bottom touches the solvent and the paper lies on the chamber wall and reaches almost to the top of the container.
- The container is closed with a cover glass or any other lid and is left for a few minutes to let the solvent vapors ascend the filter paper and saturate the air in the chamber. (Failure to saturate the chamber will result in poor separation and non-reproducible results).
- The TLC plate is then placed in the chamber so that the spot(s) of the sample do not touch the surface of the eluent in the chamber, and the lid is closed. The solvent moves up the plate by capillary action, meets the sample mixture and carries it up the plate (elutes the sample).
- The plate should be removed from the chamber before the solvent front reaches the top of the stationary phase (continuation of the elution will give a misleading result) and dried.
- Without delay, the *solvent front*, the furthest extent of solvent up the plate, is marked.
- The plate is visualised. As some plates are pre-coated with a phosphor such as zinc sulfide, allowing many compounds to be visualized by using ultraviolet light; dark spots appear where the compounds block the UV light from striking the plate. Alternatively, plates can be sprayed or immersed in chemicals after elution. Various visualising agents react with the spots to produce visible results.

3.1.3 Separation Process and Principle

Different compounds in the sample mixture travel at different rates due to the differences in their attraction to the stationary phase and because of differences in solubility in the solvent. By changing the solvent, or perhaps using a mixture, the separation of components (measured by the R_f value) can be adjusted. Also, the separation achieved with a TLC plate can be used

to estimate the separation of a flash chromatography column. (A compound elutes from a column when the amount of solvent collected is equal to $1/R_f$.) Chemists often use TLC to develop a protocol for separation by chromatography and use TLC to determine which fractions contain the desired compounds.

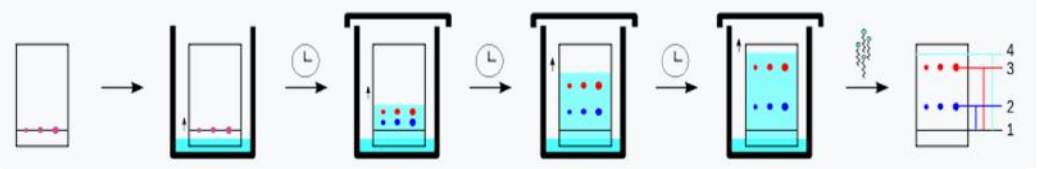


Fig 15:Development of a TLC plate, a purple spot separates into a red and blue spot

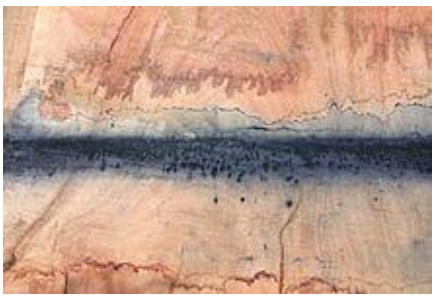


Fig. 16: Surface of a freshly cut Plank

Surface of a freshly cut plank of *Eucalyptus camaldulensis* displaying thin-layer chromatography. The horizontal blue strip is from a reaction between the iron band saw supports and the acidic timber

3.1.4 Analysis of TLC

As the chemicals being separated may be colorless, several methods exist to visualise the spots:

- fluorescent analytes like quinine may be detected under blacklight (366 nm)
- Often a small amount of a fluorescent compound, usually manganese-activated zinc silicate, is added to the adsorbent that allows the visualisation of spots under UV-C light (254 nm). The adsorbent layer will thus fluoresce light-green by itself, but spots of analyte quench this fluorescence.
- Iodine vapors are a general unspecific color reagent

- Specific color reagents into which the TLC plate is dipped or which are sprayed onto the plate exist.
  - Potassium permanganate – oxidation

- Bromine

- In the case of lipids, the chromatogram may be transferred to a PVDF membrane and then subjected to further analysis, for example mass spectrometry, a technique known as Far-Eastern blotting.
  Once visible, the R_f value, or retardation factor, of each spot can be determined by dividing the distance the product traveled by the distance the solvent front traveled using the initial spotting site as reference. These values depend on the solvent used and the type of TLC plate and are not physical constants.

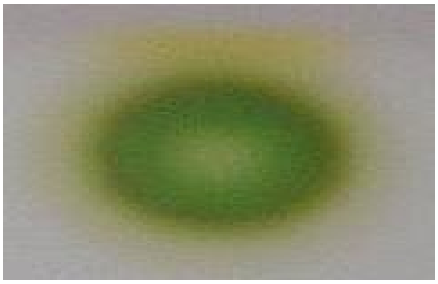
3.1.5 Application

1. Characterisation

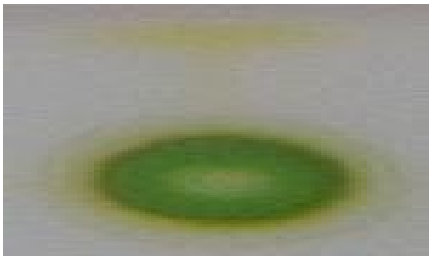
- **In organic chemistry, crop and animal science laboratories** reactions are qualitatively monitored with TLC. Spots sampled with a capillary tube are placed on the plate: a spot of starting material, a spot from the reaction mixture, and a cross-spot with both. A small (3 by 7 cm) TLC plate takes a couple of minutes to run. The analysis is qualitative, and it will show if the starting material has disappeared, i.e. the reaction is complete, if any product has appeared, and how many products are generated (although this might be underestimated due to co-elution). Unfortunately, TLCs from low-temperature reactions may give misleading results, because the sample is warmed to room temperature in the capillary, which can alter the reaction—the warmed sample analysed by TLC is not the same as what is in the low-temperature flask. One such reaction is the DIBALH reduction of ester to aldehyde.
- **In one study TLC has been applied in the screening of organic reactions**, for example in the fine-tuning of BINAP synthesis from 2-naphthol. In this method, the alcohol and catalyst solution (for instance iron(III) chloride) are placed separately on the base line, then reacted, and then instantly analysed.
- **A special application of TLC is in the characterisation of radiolabeled compounds,** where it is used to determine radiochemical purity. The TLC sheet is visualised using a sheet of photographic film or an instrument capable of measuring radioactivity. It may be visualised using other means as well. This method is much more sensitive than the others and can be used to detect an extremely small amount of a compound, provided that it carries a radioactive atom.

iv. Isolation

Since different compounds will travel a different distance in the stationary phase, chromatography can be used to isolate components of a mixture for further analysis. The separated compounds each occupying a specific area on the plate, they can be scraped off (along with the stationary phase particles) and dissolved into an appropriate solvent. As an example, in the chromatography of an extract of green plant material (for example spinach) shown in seven stages of development, Carotene elutes quickly and is only visible until step 2. Chlorophyll A and B are halfway in the final step and lutein the first compound staining yellow. Once the chromatography is over, the carotene can be removed from the plate, extracted into a solvent and placed into a spectrophotometer to determine its spectrum. The quantities extracted are small and a technique such as column chromatography is preferred to separate larger amounts.



Step 1



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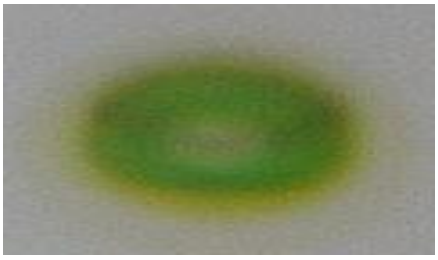

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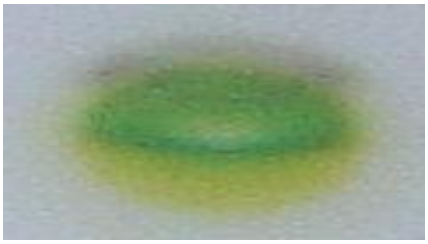

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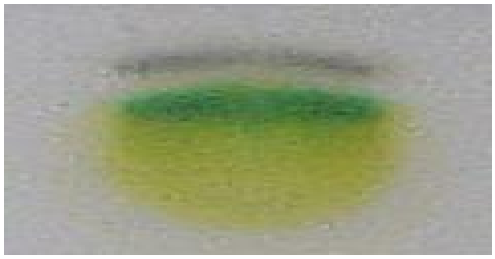

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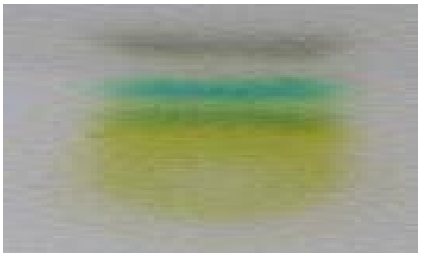

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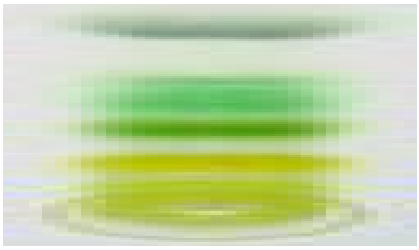

Step 7
Fig. 17

v. Examining reactions

TLC is also used for the identification of the completion of any chemical reaction. To determine this it is observed that at the beginning of a reaction the entire spot is occupied by the starting chemicals or materials on the plate. As the reaction starts taking place the spot formed by the initial chemicals starts reducing and eventually replaces the whole spot of starting chemicals with a new product present on the plate. The formation of an entirely new spot determines the completion of a reaction.

4.0 CONCLUSION

Separation of compounds is based on the competition of the solute and the mobile phase for binding sites on the stationary phase. For instance, if normal-phase silica gel is used as the stationary phase, it can be considered polar. Given two compounds that differ in polarity, the more polar compound has a stronger interaction with the silica and is, therefore, better able to displace the mobile phase from the available binding sites. As a consequence, the less polar compound moves higher up the plate (resulting in a higher R_f value). If the mobile phase is changed to a more polar solvent or mixture of solvents, it becomes better at binding to the polar plate and therefore displacing solutes from it, so all compounds on the TLC plate will move higher up the plate.

5.0 SUMMARY

Thin-layer chromatography (TLC) is a chromatography technique used to separate non-volatile mixtures. Thin-layer chromatography is normally carried out on a sheet of glass, plastic, or aluminum foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminum oxide (alumina), or cellulose. This layer of adsorbent is known as the stationary phase. After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action.

6.0 TUTOR-MARKED ASSIGNMENT

1. Explain in details the term “Thin Layer Chromatography” (TLC)
2. Briefly explain preparation of plate in TLC.
3. To run a thin layer chromatography plate, state the seven procedures involved.
4. In Analysis of TLC, state at least five methods of visualising the spots.
5. State and briefly explain the 3 major applications of TLC.

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UNIT 5 GAS CHROMATOGRAPHY (GC)

CONTENTS

1.0 INTRODUCTION

Gas chromatography (**GC**) is a common type of chromatography used in analytical chemistry for separating and analyzing compounds that can be vaporized without decomposition. Main uses of GC involve testing the purity of a particular substance, and also separating the different components of a mixture (the relative amounts of such components can also be determined). In some situations, GC may help in identifying a compound.

2.0 OBLECTIVES

By the end of this unit, you will be able to:

- know what gas chromatography is all about
- understand how gas chromatography functions.

3.0 MAIN CONTENT

3.1 Gas Chromatography

Gas chromatography is in principle similar to other forms of chromatography, such as HPLC, TLC), but has some notable differences. First, the process of separating the compounds in a mixture is carried out between a liquid stationary phase and a gas mobile phase, whereas in others, the stationary phase is a solid and the mobile phase is a liquid. (Hence the full name of the procedure is "Gas–liquid chromatography", referring to the mobile and stationary phases, respectively.) Second, the column through which the gas phase passes is located in an oven where the

temperature of the gas can be controlled, whereas other chromatography (typically) has no such temperature control. Gas chromatography is also sometimes known as **vapor-phase chromatography** (VPC), or **gas–liquid partition chromatography**(GLPC). These alternative names, as well as their respective abbreviations, are frequently used in scientific literature. In gas chromatography, the *mobile phase* (or "moving phase") is a carrier gas, usually an inert gas such as helium or an unreactive gas such as nitrogen. Helium remains the most commonly used carrier gas in about 90% of instruments although hydrogen is preferred for improved separations.[3] The *stationary phase* is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called a column (an homage to the fractionating column used in distillation). The instrument used to perform gas chromatography is called a *gas chromatograph* (or "aerograph", "gas separator").

Fig. 18: A gas chromatograph with a headspace sampler

3.1.1 GC Analysis

A gas chromatograph is a chemical analysis instrument for separating chemicals in a complex sample. A gas chromatograph uses a flow-through narrow tube known as the *column*, through which different chemical constituents of a sample pass in a gas stream (carrier gas, *mobile phase*) at different rates depending on their various chemical and physical properties and their interaction with a specific column filling, called the *stationary phase*. As the chemicals exit the end of the column, they are detected and identified electronically. The function of the stationary phase in the column is to separate different components, causing each one to exit the column at a different time (*retention time*). Other parameters that can be used to alter

the order or time of retention are the carrier gas flow rate, column length and the temperature.

3.1.1.1 Analytical Procedure

- In a GC analysis, a known volume of gaseous or liquid analyte is injected into the "entrance" (head) of the column, usually using a microsyringe (or, solid phase microextraction fibers, or a gas source switching system).
- As the carrier gas sweeps the analyte molecules through the column, this motion is inhibited by the adsorption of the analyte molecules either onto the column walls or onto packing materials in the column.
- The rate at which the molecules progress along the column depends on the strength of adsorption, which in turn depends on the type of molecule and on the stationary phase materials.
- Since each type of molecule has a different rate of progression, the various components of the analyte mixture are separated as they progress along the column and reach the end of the column at different times (retention time).
- A detector is used to monitor the outlet stream from the column; thus, the time at which each component reaches the outlet and the amount of that component can be determined.
- Generally, substances are identified (qualitatively) by the order in which they emerge (elute) from the column and by the retention time of the analyte in the column.

3.1.2 Components of GC

1. Autosamplers

The auto sampler provides the means to introduce a sample automatically into the inlets. Manual insertion of the sample is possible but is no longer common. Automatic insertion provides better reproducibility and time-optimisation. Different kinds of auto samplers exist. Auto samplers can be classified in relation to sample capacity (auto-injectors vs. auto samplers, where auto-injectors can work a small number of samples), to robotic technologies (XYZ robot vs. rotating robot – the most common), or to analysis:

- Liquid
- Static head-space by syringe technology
- Dynamic head-space by transfer-line technology

- Solid phase microextraction (SPME)

2. Inlets

Split/splitless inlet.
The column inlet (or injector) provides the means to introduce a sample into a continuous flow of carrier gas. The inlet is a piece of hardware attached to the column head.

Common inlet types are:

- S/SL (split/splitless) injector; a sample is introduced into a heated small chamber via a syringe through a septum – the heat facilitates volatilization of the sample and sample matrix. The carrier gas then either sweeps the entirety (splitless mode) or a portion (split mode) of the sample into the column.
- On-column inlet; the sample is here introduced directly into the column in its entirety without heat, or at a temperature below the boiling point of the solvent. The low temperature condenses the sample into a narrow zone. The column and inlet can then be heated, releasing the sample into the gas phase. This ensures the lowest possible temperature for chromatography and keeps samples from decomposing above their boiling point.
- PTV injector; Temperature-programmed sample introduction was first described by Vogt in 1979. Originally Vogt developed the technique as a method for the introduction of large sample volumes (up to 250 µL) in capillary GC. Vogt introduced the sample into the liner at a controlled injection rate. The temperature of the liner was chosen slightly below the boiling point of the solvent. The low-boiling solvent was continuously evaporated and vented through the split line. By introducing the sample at a low initial liner temperature many of the disadvantages of the classic hot injection techniques could be circumvented.
- Gas source inlet or gas switching valve; gaseous samples in collection bottles are connected to what is most commonly a six-port switching valve. The carrier gas flow is not interrupted while a sample can be expanded into a previously evacuated sample loop. Upon switching, the contents of the sample loop are inserted into the carrier gas stream.
- P/T (Purge-and-Trap) system; An inert gas is bubbled through an aqueous sample causing insoluble volatile chemicals to be purged from the matrix. The volatiles are 'trapped' on an absorbent column (known as a trap or concentrator) at ambient temperature. The trap is then heated and the volatiles are directed into the carrier gas stream.

Samples requiring preconcentration or purification can be introduced via such a system, usually hooked up to the S/SL port.

The choice of carrier gas (mobile phase) is important. Hydrogen has a range of flow rates that are comparable to helium in efficiency. However, helium may be more efficient and provide the best separation if flow rates are optimized. Helium is non-flammable and works with a greater number of detectors and older instruments. Therefore, helium is the most common carrier gas used. However, the price of helium has gone up considerably over recent years, causing an increasing number of chromatographers to switch to hydrogen gas. Historical use, rather than rational consideration, may contribute to the continued preferential use of helium.

3. Detectors

The most commonly used detectors are the flame ionization detector (FID) and the thermal conductivity detector (TCD). Both are sensitive to a wide range of components, and both work over a wide range of concentrations. While TCDs are essentially universal and can be used to detect any component other than the carrier gas (as long as their thermal conductivities are different from that of the carrier gas, at detector temperature), FIDs are sensitive primarily to hydrocarbons, and are more sensitive to them than TCD. However, a FID cannot detect water. Both detectors are also quite robust. Since TCD is non-destructive, it can be operated in-series before a FID (destructive), thus providing complementary detection of the same analytes. Other detectors are sensitive only to specific types of substances, or work well only in narrower ranges of concentrations.

i. **Thermal Conductivity Detector (TCD):** relies on the thermal conductivity of matter passing around a tungsten -rhenium filament with a current traveling through it. In this set up helium or nitrogen serve as the carrier gas because of their relatively high thermal conductivity which keep the filament cool and maintain uniform resistivity and electrical efficiency of the filament. However, when analyte molecules elute from the column, mixed with carrier gas, the thermal conductivity decreases and this causes a detector response.
ii. **Flame Ionisation Detector (FID)**: In this detector, electrodes are placed adjacent to a flame fueled by hydrogen / air near the exit of the column, and when carbon containing compounds exit the column they are pyrolyzed by the flame. This detector works only for organic / hydrocarbon containing compounds due to the ability of

the carbons to form cations and electrons upon pyrolysis which generates a current between the electrodes.

3.1.3 Methods

The method is the collection of conditions in which the GC operates for a given analysis. Method development is the process of determining what conditions are adequate and/or ideal for the analysis required.Some GCs also include valves which can change the route of sample and carrier flow. The timing of the opening and closing of these valves can be important to method development. They include:

i. Carrier gas selection and flow rates
ii. Stationary compound selection
iii. Inlet types and flow rates

3.1.4 Sample Size and Injection Technique

- **Sample injection**
  The real chromatographic analysis starts with the introduction of the sample onto the column. The development of capillary gas chromatography resulted in many practical problems with the injection technique. The technique of on-column injection, often used with packed columns, is usually not possible with capillary columns. In the injection system in the capillary gas chromatograph the amount injected should not overload the column and the width of the injected plug should be small compared to the spreading due to the chromatographic process.
- **Column selection**
  The choice of column depends on the sample and the active measured. The main chemical attribute regarded when choosing a column is the polarity of the mixture, but functional groups can play a large part in column selection. The polarity of the sample must closely match the polarity of the column stationary phase to increase resolution and separation while reducing run time. The separation and run time also depends on the film thickness (of the stationary phase), the column diameter and the column length.

3.1.5 Data Reduction and Analysis

- **Qualitative analysis**
  Generally, chromatographic data is presented as a graph of detector response (y-axis) against retention time (x-axis), which is called a

chromatogram. This provides a spectrum of peaks for a sample representing the analytes present in a sample eluting from the column at different times. Retention time can be used to identify analytes if the method conditions are constant. Also, the pattern of peaks will be constant for a sample under constant conditions and can identify complex mixtures of analytes. However, in most modern applications, the GC is connected to a mass spectrometer or similar detector that is capable of identifying the analytes represented by the peaks.

- **Quantitative analysis**
  The area under a peak is proportional to the amount of analyte present in the chromatogram. By calculating the area of the peak using the mathematical function of integration, the concentration of an analyte in the original sample can be determined. Concentration can be calculated using a calibration curve created by finding the response for a series of concentrations of analyte, or by determining the relative response factor of an analyte. The relative response factor is the expected ratio of an analyte to an internal standard (or external standard) and is calculated by finding the response of a known amount of analyte and a constant amount of internal standard (a chemical added to the sample at a constant concentration, with a distinct retention time to the analyte).

In most modern GC-MS systems, computer software is used to draw and integrate peaks, and match MS spectra to library spectra.

3.1.6 Applications

In general, substances that vaporize below 300 °C (and therefore are stable up to that temperature) can be measured quantitatively. The samples are also required to be salt-free; they should not contain ions. Very minute amounts of a substance can be measured, but it is often required that the sample must be measured in comparison to a sample containing the pure, suspected substance known as a reference standard.

- Various temperature programs can be used to make the readings more meaningful; for example to differentiate between substances that behave similarly during the GC process.
- Professionals working with GC analyze the content of a chemical product, for example in assuring the quality of products in the chemical industry; or measuring toxic substances or pollutions in soil, air or water. GC is very accurate if used properly and can

measure picomoles of a substance in a 1 ml liquid sample, or parts-per-billion concentrations in gaseous samples.

- The hydrocarbons are separated using a capillary column and detected with a FID. A complication with light gas analyses that include H_2 is that He, which is the most common and most sensitive inert carrier (sensitivity is proportional to molecular mass) has an almost identical thermal conductivity to hydrogen (it is the difference in thermal conductivity between two separate filaments in a Wheatstone Bridge type arrangement that shows when a component has been eluted).
- Argon is often used when analyzing gas phase chemistry reactions such as F-T synthesis so that a single carrier gas can be used rather than two separate ones. The sensitivity is reduced, but this is a trade off for simplicity in the gas supply.

4.0 CONCLUSION

A gas chromatograph uses a flow-through narrow tube known as the *column*, through which different chemical constituents of a sample pass in a gas stream (carrier gas, *mobile phase*) at different rates depending on their various chemical and physical properties and their interaction with a specific column filling, called the *stationary phase*. As the chemicals exit the end of the column, they are detected and identified electronically.

5.0 SUMMARY

Gas chromatography (**GC**) is a common type of chromatography used in analytical chemistry for separating and analyzing compounds that can be vaporized without decomposition. Main uses of GC involve testing the purity of a particular substance, and also separating the different components of a mixture (the relative amounts of such components can also be determined).

6.0 TUTOR-MARKED ASSIGNMENT

1. What do you understand by gas chromatography and what is the difference between gas chromatography and HPLC and TLC
2. Discuss briefly, the principles and operations of Gas Chromatography
3. State the analytical procedures of gas chromatography
4. Discuss briefly, the following components of GC
   a. Autosamplers
   b. Inlets

c. Detectors

5. outline the three methods in Gas Chromatography analysis

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UNIT 6 POLYMERASE CHAIN REACTION (PCR)

CONTENTS

1.0 INTRODUCTION

Polymerase chain reaction (PCR) is a technique used in the lab to make millions of copies of a particular section of DNA. It was first developed in the 1980s. Using PCR, copies of DNA sequences are exponentially amplified to generate thousands to millions of more copies of that particular DNA segment. PCR is now a common and often indispensable technique used in many laboratory research for a broad variety of applications including biomedical research and criminal forensics. Agriculturally, researches in animal DNAs can be carried out with the aid of PCR, which would find great use in animal breeding and genetics.

PCR was developed by Kary Mullis in 1983 while he was an employee of the Certus Corporation. He was awarded the Nobel Prize in Chemistry in 1993 (along with Michael Smith) for his work in developing the method.The vast majority of PCR methods rely on thermal cycling. Thermal cycling exposes reactants to repeated cycles of heating and cooling to permit different temperature-dependent reactions—specifically, DNA melting and enzyme-driven DNA replication. PCR employs two main reagents – primers (which are short single strand DNA fragments known as oligonucleotides that are a complementary sequence to the target DNA region) and a DNA polymerase.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- know the principles and operations of PCR

- use PCR in the laboratories to carry out analysis.

3.0 MAIN CONTENT

3.1 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) was originally developed in 1983 by the American biochemist Kary Mullis. He was awarded the Nobel Prize in Chemistry in 1993 for his pioneering work. PCR is used in molecular biology to make many copies of (amplify) small sections of DNA or a gene. Using PCR it is possible to generate thousands to millions of copies of a particular section of DNA from a very small amount of DNA.PCR is a common tool used in medical, biological and animal science research labs. It is used in the early stages of processing DNA for sequencing, for detecting the presence or absence of a gene to help identify pathogens during infection, and when generating forensic DNA profiles from tiny samples of DNA.

3.1.2 Application of PCR

Applications of the PCR technique include:

i. DNA cloning for sequencing, gene cloning and manipulation .
ii. Gene mutagenesis: PCR can be used to create mutant genes with mutations chosen by scientists at will. These mutations can be chosen in order to understand how proteins accomplish their functions, and to change or improve protein function.
iii. Construction of DNA-based phylogenies, or functional analysis of genes.
iv. Diagnosis and monitoring of hereditary diseases.
v. Amplification of ancient DNA: An exciting application of PCR is the phylogenic analysis of DNA from *ancient sources*, such as that found in the recovered bones of Neanderthals, from frozen tissues of mammoths, or from the brain of Egyptian mummies.
vi. Analysis of genetic fingerprints for DNA profiling (for example, in forensic science and parentage testing).
vii. Detection of pathogens in nucleic acid tests for the diagnosis of infectious diseases.

Fig. 19: A typical PCR Machine

3.1.3 The principles behind PCR

- PCR amplifies a specific region of a DNA strand (the DNA target). The amount of amplified product is determined by the available substrates in the reaction, which become limiting as the reaction progresses. Most PCR methods amplify DNA fragments of between 0.1 and 10 kilo base pairs (kbp) in length, although some techniques allow for amplification of fragments up to 40 kbp.
- A basic PCR set-up requires several components and reagents, including:

  i. a *DNA template* that contains the DNA target region to amplify;
  ii. a *DNA polymerase*; an enzyme that polymerizes new DNA strands; heat-resistant Taq polymerase is especially common, as it is more likely to remain intact during the high-temperature DNA denaturation process;
  iii. two DNA *primers* that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strands of the DNA target (DNA polymerase can only bind to and elongate from a double-stranded region of DNA; without primers there is no double-stranded initiation site at which the polymerase can bind);specific primers that are complementary to the DNA target region are selected beforehand, and are often custom-made in a laboratory or purchased from commercial biochemical suppliers; *deoxynucleoside triphosphates*, or

dNTPs (sometimes called "deoxynucleotide triphosphates"; nucleotides containing triphosphate groups).

iv. The reaction is commonly carried out in a volume of 10–200 μL in small reaction tubes (0.2–0.5 mL volumes) in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction.

The principles behind every PCR, whatever the sample of DNA, are the same.

3.1.4 Five Core 'Ingredients' Required To Set Up a PCR Are:

- the DNA template to be copied
- primers which are short stretches of DNA that initiate the PCR reaction, designed to bind to either side of the section of DNA you want to copy.
- DNA nucleotide bases (also known as dNTPs).
- DNA bases (A, C, G and T) are the building blocks of DNA and are needed to construct the new strand of DNA.
- Taq polymerase enzyme to add in the new DNA bases buffer to ensure the right conditions for the reaction.

PCR involves a process of heating and cooling called thermal cycling which is carried out by machine.

3.1.5 There are Three Main Stages of PCR

Denaturing – when the double-stranded template DNA is heated to separate it into two single strands.During this stage the cocktail containing the template DNA and all the other core ingredients is heated to 94-95^{0}C.The high temperature causes the hydrogen bonds between the bases in two strands of template DNA to break and the two strands to separate.

Annealing – when the temperature is lowered to enable the DNA primers to attach to the template DNA.During this stage the reaction is cooled to 50-65^{0}C. This enables the primers to attach to a specific location on the single-stranded template DNA by way of hydrogen bonding (the exact temperature depends on the melting temperature of the primers you are using).

Extending – when the temperature is raised and the new strand of DNA is made by the Taq polymerase enzyme. During this final step, the heat is increased to 72^0C to enable the new DNA to be made by a special Taq DNA polymerase enzyme which adds DNA bases.Taq DNA polymerase is an enzyme taken from the heat-loving bacteria *Thermusaquaticus*. This bacterium lives in hot springs so can tolerate temperatures above 80^0C.The bacteria's DNA polymerase is very stable at high temperatures, which means it can withstand the temperatures needed to break the strands of DNA apart in the denaturing stage of PCR.

These three stages are repeated 20-40 times, doubling the number of DNA copies each time. A complete PCR reaction can be performed in a few hours, or even less than an hour with certain high-speed machines. After PCR has been completed, a method called electrophoresis can be used to check the quantity and size of the DNA fragments produced. Fig. 20. shows how the polymerase chain reaction (PCR) produces lots of copies of DNA.

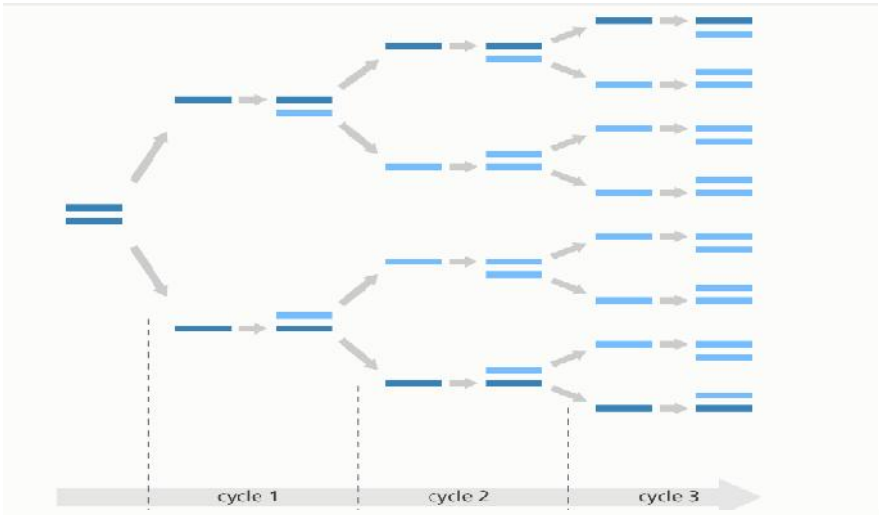

Fig. 20. Illustration showing how the polymerase chain reaction (PCR) produces lots of copies of DNA. Source: Genome Research Limited

3.1.6 Stages

As with other chemical reactions, the reaction rate and efficiency of PCR are affected by limiting factors. Thus, the entire PCR process can further be divided into three stages based on reaction progress:

- **Exponential amplification:** At every cycle, the amount of product is doubled (assuming 100% reaction efficiency). After 30 cycles, a single copy of DNA can be increased up to 1 000 000 000 (one

billion) copies. In a sense, then, the replication of a discrete strand of DNA is being manipulated in a tube under controlled conditions.The reaction is very sensitive: only minute quantities of DNA must be present.

- **Leveling off stage:** The reaction slows as the DNA polymerase loses activity and as consumption of reagents such as dNTPs and primers causes them to become limiting.
- **Plateau:** No more product accumulates due to exhaustion of reagents and enzyme.

3.1.7 Advantages

PCR has a number of advantages.

i. It is fairly simple to understand and to use,
ii. It produces results rapidly.
iii. The technique is highly sensitive with the potential to produce millions to billions of copies of a specific product for sequencing, cloning, and analysis.
iv. PCR is a very powerful and practical research tool. The sequencing of unknown etiologies of many diseases are being figured out by the PCR.
v. The PCR technique can help identify the sequence of previously unknown viruses related to those already known and thus give us a better understanding of the disease itself.

3.1.8 Limitations

i. One major limitation of PCR is that prior information about the target sequence is necessary in order to generate the primers that will allow its selective amplification. This means that, typically, PCR users must know the precise sequence(s) upstream of the target region on each of the two single-stranded templates in order to ensure that the DNA polymerase properly binds to the primer-template hybrids and subsequently generates the entire target region during DNA synthesis.
ii. Like all enzymes, DNA polymerases are also prone to error, which in turn causes mutations in the PCR fragments that are generated.
iii. Another limitation of PCR is that even the smallest amount of contaminating DNA can be amplified, resulting in misleading or ambiguous results.

3.1.9 Variations of PCR

1. **Allele-specific PCR:** a diagnostic or cloning technique based on single-nucleotide variations (SNVs not to be confused with SNPs) (single-base differences in a patient).

2. **Assembly PCR or Polymerase Cycling Assembly (PCA):** artificial synthesis of long DNA sequences by performing PCR on a pool of long oligonucleotides with short overlapping segments.
3. **Asymmetric PCR:** preferentially amplifies one DNA strand in a double-stranded DNA template. It is used in sequencing and hybridization probing where amplification of only one of the two complementary strands is required.
4. **Convective PCR:** a pseudo-isothermal way of performing PCR. Instead of repeatedly heating and cooling the PCR mixture, the solution is subjected to a thermal gradient. The resulting thermal instability driven convective flow automatically shuffles the PCR reagents from the hot and cold regions repeatedly enabling PCR
5. **Dial-out PCR:** a highly parallel method for retrieving accurate DNA molecules for gene synthesis. A complex library of DNA molecules is modified with unique flanking tags before massively parallel sequencing. Tag-directed primers then enable the retrieval of molecules with desired sequences by PCR.
6. **Digital PCR (dPCR):** used to measure the quantity of a target DNA sequence in a DNA sample. The DNA sample is highly diluted so that after running many PCRs in parallel, some of them do not receive a single molecule of the target DNA. The target DNA concentration is calculated using the proportion of negative outcomes. Hence the name 'digital PCR'.
7. **Helicase-dependent amplification:** similar to traditional PCR, but uses a constant temperature rather than cycling through denaturation and annealing/extension cycles. DNA helicase, an enzyme that unwinds DNA, is used in place of thermal denaturation.
8. **Hot start PCR:** a technique that reduces non-specific amplification during the initial set up stages of the PCR. It may be performed manually by heating the reaction components to the denaturation temperature (e.g., 95 °C) before adding the polymerase.
9. **In silico PCR** (digital PCR, virtual PCR, electronic PCR, e-PCR) refers to computational tools used to calculate theoretical polymerase chain reaction results using a given set of primers (probes) to amplify DNA sequences from a sequenced genome or transcriptome. In silico PCR was proposed as an educational tool for molecular biology.

10. **Intersequence-specific PCR (ISSR):** a PCR method for DNA fingerprinting that amplifies regions between simple sequence repeats to produce a unique fingerprint of amplified fragment lengths.

4.0 CONCLUSION

Polymerase chain reaction (PCR) is a technique used in the lab to make millions of copies of a particular section of DNA. Using PCR, copies of DNA sequences are exponentially amplified to generate thousands to millions of more copies of that particular DNA segment. PCR is now a common and often indispensable technique used in many laboratory researches for a broad variety of applications including biomedical research and criminal forensics. Agriculturally, researches in animal DNAs can be carried out with the aid of PCR, which would find great use in animal breeding and genetics.

5.0 SUMMARY

The polymerase chain reaction (PCR) was originally developed in 1983 by the American biochemist Kary Mullis. PCR is used in molecular biology to make many copies of (amplify) small sections of DNAor a gene. Using PCR it is possible to generate thousands to millions of copies of a particular section of DNA from a very small amount of DNA.PCR is a common tool used in medical, biological and animal science research labs. It is used in the early stages of processing DNA for sequencing, for detecting the presence or absence of a gene to help identify pathogens during infection, and when generating forensic DNA profiles from tiny samples of DNA.

6.0 TUTOR-MARKED ASSIGNMENT

1. What is the main use of PCRs and where can they be found?
2. Outline eight (8) applications of PCR
3. Briefly explain the operating principles of PCR
4. Discuss these major stages of PCR
   a. Denaturing
   b. Annealing and
   c. Extending
5. State five (5) advantages and three (3) limitations of PCR
6. Mention and briefly explain at least five (5) variations of PCR

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UNIT 7 GEL ELECTROPHORESIS

CONTENTS

1.0 INTRODUCTION

Gel electrophoresis is a method normally employed in the separation and analysis of macromolecules such as (DNA, RNA and proteins) as well as their fragments, based on their size and charge. It is employed in clinical chemistry and animal science laboratories to separate proteins by charge or size (IEF agarose, essentially size independent) and in biochemistry and molecular biology to separate a mixed population of DNA and RNA fragments by length, to estimate the size of DNA and RNA fragments or to separate proteins by charge. Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through a matrix of agarose or other substances. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. This phenomenon is called sieving. Proteins are separated by charge in agarose because the pores of the gel are too large to sieve proteins. Gel electrophoresis can also be used for separation of nanoparticles.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- understand what gel electrophoresis is all about
- know the gel materials used in gel electrophoresis.

3.0 MAIN CONTENT

3.1 Gel Electrophoresis

In simple terms, electrophoresis is a process which enables the sorting of molecules based on size. Using an electric field, molecules (such as DNA) can be made to move through a gel made of agarose or polyacrylamide.Gel electrophoresis uses a gel as an anticonvective medium or sieving medium during electrophoresis, the movement of a charged particle in an electrical field. Gels suppress the thermal convection caused by application of the electric field, and can also act as a sieving medium, retarding the passage of molecules; gels can also simply serve to maintain the finished separation, so that a post electrophoresis stain can be applied. DNA Gel electrophoresis is usually performed for analytical purposes, often after amplification of DNA via polymerase chain reaction (PCR), but may be used as a preparative technique prior to use of other methods such as mass spectrometry, RFLP, PCR, cloning, DNA sequencing, or Southern blotting for further characterisation.

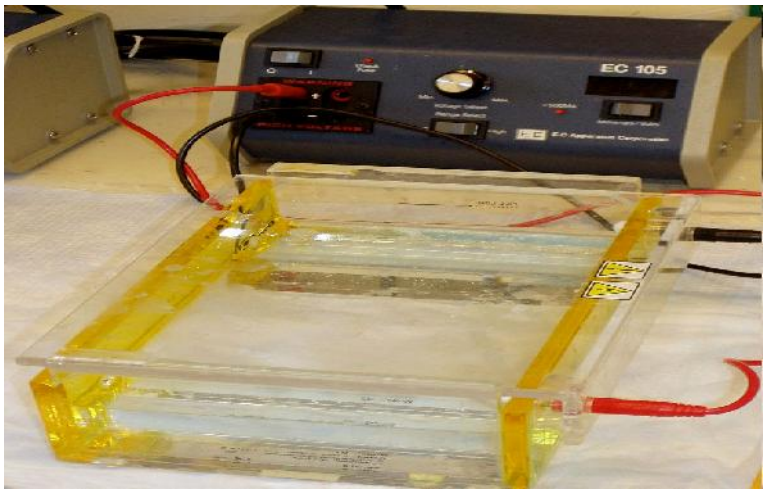

Fig. 21. Gel Electrophoresis Machine

3.1.1 Types of Gel

Agarose and polyacrylamide gels are most typically used. This is because each type of gel is well-suited to different types and sizes of analyte. While polyacrylamide gels are usually used for proteins because of thier very high resolving power for small fragments of DNA (5-500 bp), agarose gels on the other hand have lower resolving power for DNA but have greater range of separation, and are therefore used for DNA fragments of usually 50-20,000 bp in size, but resolution of over 6 Mb is possible with pulsed field

gel electrophoresis (PFGE). Polyacrylamide gels are run in a vertical configuration while agarose gels are typically run horizontally in a submarine mode. They also differ in their casting methodology, as agarose sets thermally, while polyacrylamide forms in a chemical polymerisation reaction.

1. **Agarose**

   Agarose gels are made from the natural polysaccharide polymers extracted from seaweed. Agarose gels are easily cast and handled compared to other matrices, because the gel setting is a physical rather than chemical change. Samples are also easily recovered. After the experiment is finished, the resulting gel can be stored in a plastic bag in a refrigerator.

   Agarose gels do not have a uniform pore size, but are optimal for electrophoresis of proteins that are larger than 200 kDa.Agarose gel electrophoresis can also be used for the separation of DNA fragments ranging from 50 base pair to several megabases (millions of bases), the largest of which require specialized apparatus. The distance between DNA bands of different lengths is influenced by the percent agarose in the gel, with higher percentages requiring longer run times, sometimes days. Instead high percentage agarose gels should be run with a pulsed field electrophoresis (PFE), or field inversion electrophoresis.

2. **Polyacrylamide**

   Polyacrylamide gel electrophoresis (PAGE) is used for separating proteins ranging in size from 5 to 2,000 kDa due to the uniform pore size provided by the polyacrylamide gel. Pore size is controlled by modulating the concentrations of acrylamide and bis-acrylamide powder used in creating a gel. Care must be used when creating this type of gel, as acrylamide is a potent neurotoxin in its liquid and powdered forms.

   Traditional DNA sequencing techniques such as Maxam-Gilbert or Sanger methods used polyacrylamide gels to separate DNA fragments differing by a single base-pair in length so the sequence could be read. Most modern DNA separation methods now use agarose gels, except for particularly small DNA fragments. It is currently most often used in the field of immunology and protein analysis, often used to separate different proteins or isoforms of the same protein into separate bands. These can be transferred onto a nitrocellulose or PVDF membrane to be probed with antibodies and corresponding markers, such as in a western blot.

3. Starch

Partially hydrolysed potato starch makes for another non-toxic medium for protein electrophoresis. The gels are slightly more opaque than acrylamide or agarose. Non-denatured proteins can be separated according to charge and size. They are visualised using Napthal Black or Amido Black staining. Typical starch gel concentrations are 5% to 10%.

3.1.2 Gel Conditions

1. Denaturing

- Denaturing gels are run under conditions that disrupt the natural structure of the analyte, causing it to unfold into a linear chain.
- Thus, the mobility of each macromolecule depends only on its linear length and its mass-to-charge ratio.
- Thus, the secondary, tertiary, and quaternary levels of biomolecular structure are disrupted, leaving only the primary structure to be analysed.
- Nucleic acids are often denatured by including urea in the buffer, while proteins are denatured using sodium dodecyl sulfate, usually as part of the SDS-PAGE process.
- For full denaturation of proteins, it is also necessary to reduce the covalent disulfide bonds that stabilise their tertiary and quaternary structure, a method called reducing PAGE.
- Reducing conditions are usually maintained by the addition of beta-mercaptoethanol or dithiothreitol. For general analysis of protein samples, reducing PAGE is the most common form of protein electrophoresis.

2. Native

- Native gels are run in non-denaturing conditions, so that the analyte's natural structure is maintained.
- Unlike denaturing methods, native gel electrophoresis does not use a charged denaturing agent.
- The molecules being separated (usually proteins or nucleic acids) therefore differ not only in molecular mass and intrinsic charge, but also the cross-sectional area, and thus experience different electrophoretic forces dependent on the shape of the overall structure.
- This allows the physical size of the folded or assembled complex to affect the mobility, allowing for analysis of all four levels of the biomolecular structure.

- For biological samples, detergents are used only to the extent that they are necessary to lyse lipid membranes in the cell.
- Complexes remain—for the most part—associated and folded as they would be in the cell.
- One downside, however, is that complexes may not separate cleanly or predictably, as it is difficult to predict how the molecule's shape and size will affect its mobility. Addressing and solving this problem is a major aim of quantitative native PAGE.
- For proteins, since they remain in the native state they may be visualised not only by general protein staining reagents but also by specific enzyme-linked staining.
- Native gel electrophoresis is typically used in proteomics and metallomics. However, native PAGE is also used to scan genes (DNA) for unknown mutations as in Single-strand conformation polymorphism.

3. Buffers

- Buffers in gel electrophoresis are used to provide ions that carry a current and to maintain the pH at a relatively constant value.
- These buffers have plenty of ions in them, which is necessary for the passage of electricity through them.
- Something like distilled water or benzene contains few ions, which is not ideal for the use in electrophoresis.
- There are a number of buffers used for electrophoresis. The most common being, for nucleic acids Tris/Acetate/EDTA (TAE), Tris/Borate/EDTA (TBE).
- Many other buffers have been proposed, e.g. lithium borate, which is almost never used, based on Pubmed citations (LB), iso electric histidine, pK matched goods buffers, etc.; in most cases the purported rationale is lower current (less heat) matched ion mobilities, which leads to longer buffer life.
- However, with its low conductivity, a much higher voltage could be used (up to 35 V/cm), which means a shorter analysis time for routine electrophoresis.
- As low as one base pair size difference could be resolved in 3% agarose gel with an extremely low conductivity medium (1 mM Lithium borate).
- Most SDS-PAGE protein separations are performed using a "discontinuous" (or DISC) buffer system that significantly enhances the sharpness of the bands within the gel.
- During electrophoresis in a discontinuous gel system, an ion gradient is formed in the early stage of electrophoresis that causes all of the

proteins to focus into a single sharp band in a process called isotachophoresis.

- Separation of the proteins by size is achieved in the lower, "resolving" region of the gel. The resolving gel typically has a much smaller pore size, which leads to a sieving effect that now determines the electrophoretic mobility of the proteins.

4. Visualisation

- After electrophoresis is complete, the molecules in the gel are stained in order to make them visible and the process is called visualisation.
- DNA may be visualised using ethidium bromide which, when intercalated into DNA, fluoresce under ultraviolet light.
- Protein may be visualised using silver stain or Coomassie Brilliant Blue dye.
- Other methods may also be used to visualize the separation of the mixture's components on the gel. If the molecules to be separated contain radioactivity, Photographs can be taken of gels, often using a Gel Doc system.

5. Downstream processing

- After separation, an additional separation method may then be used, such as isoelectric focusing or SDS-PAGE.
- The gel will then be physically cut, and the protein complexes extracted from each portion separately.
- Each extract may then be analysed, such as by peptide mass fingerprinting or de novo peptide sequencing after in-gel digestion. This can provide a great deal of information about the identities of the proteins in a complex.

3.1.6 Applications of Gel Electrophoresis

- Estimation of the size of DNA molecules following restriction enzyme digestion, e.g. in restriction mapping of cloned DNA.
- Analysis of PCR products, e.g. in molecular genetic diagnosis or genetic fingerprinting
- Separation of restricted genomic DNA prior to southern transfer, or of RNA prior to Northern transfer.
- Gel electrophoresis is used in forensics, molecular biology, genetic, microbiology andbiochemistry.

3.1.7 History

- 1930s – first reports of the use of sucrose for gel electrophoresis
- 1955 – introduction of starch gels, mediocre separation (Smithies)
- 1959 – introduction of acrylamide gels; disc electrophoresis (Ornstein and Davis); accurate control of parameters such as pore size and stability; and (Raymond and Weintraub)
- 1966 – first use of agar gels
- 1969 – introduction of denaturing agents especially SDS separation of protein subunit (Weber and Osborn)
- 1970 – Laemmli separated 28 components of T4 phage using a stacking gel and SDS
- 1972 – agarose gels with ethidium bromide stain
- 1975 – 2-dimensional gels (O'Farrell); isoelectric focusing then SDS gel electrophoresis
- 1977 – sequencing gels
- 1983 – pulsed field gel electrophoresis enables separation of large DNA molecules
- 1983 – introduction of capillary electrophoresis
- 2004 – introduction of a standardized time of polymerization of acrylamide gels enables clean and predictable separation of native proteins (Kastenholz).

A 1959 book on electrophoresis by Milan Bier cites references from the 1800s. However, Oliver Smithies made significant contributions. Bier states: "The method of Smithies is finding wide application because of its unique separatory power." Taken in context, Bier clearly implies that Smithies' method is an improvement.

4.0 CONCLUSION

Gel electrophoresis is a method normally employed in the separation and analysis of macromolecules such as (DNA, RNA and proteins) as well as their fragments, based on their size and charge. Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through a matrix of agarose or other substances. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. This phenomenon is called sieving.

5.0 SUMMARY

Agarose and polyacrylamide gels are most typically used. This is because each type of gel is well-suited to different types and sizes of analyte. While polyacrylamide gels are usually used for proteins because of their very high resolving power for small fragments of DNA (5-500 bp), agarose gels on the other hand have lower resolving power for DNA but have greater range of separation, and are therefore used for DNA fragments of usually 50-20,000 bp in size, but resolution of over 6 Mb is possible with pulsed field gel electrophoresis (PFGE).

6.0 TUTOR-MARKED ASSIGNMENT

1. What do you understand as Gel electrophoresis and which labs are they found?
2. State the two major types of gel used in Gel electrophoresis and what is the different between them?
3. Mention at least four different gel conditions and briefly explain each.
4. Outline the applications of gel electrophoresis.
5. State the history of the development of gel electrophoresis.

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UNIT 8 HEMATOLOGY AND SERUM BIOCHEMISTRY

CONTENTS

1.0 INTRODUCTION

Hematology, also spelled haematology, is the branch of medicine concerned with the study of the cause, prognosis, treatment, and prevention of diseases related to blood. It involves treating diseases that affect the production of blood and its components, such as blood cells, hemoglobin, blood proteins, bone marrow, platelets, blood vessels, spleen, and the mechanism of coagulation. Such diseases might include hemophilia, blood clots, other bleeding disorders and blood cancers such as leukemia, multiple myeloma, and lymphoma. The laboratory work that goes into the study of blood is frequently performed by a medical technologist or medical laboratory scientist. Serum biochemistry on the other hand refers to the chemical analysis of blood serum. A profile of tests can be combined to evaluate renal function, electrolyte metabolism, serum proteins, digestion, injury, lipids, pancreatic function, and the liver. Specialised chemistry testing can also be undertaken to assess thyroid glands and liver function.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- understand what hematology and serum biochemistry are all about
- know how some common blood diseases that can be determined in the laboratory.

3.0 MAIN CONTENT

3.1 Hematology

Hematology, also spelled haematology, is the branch of medicine concerned with the study of the cause, prognosis, treatment, and prevention of diseases related to blood. Physicians specialized in hematology are known as **hematologists** or **haematologists**. Their routine work mainly includes the care and treatment of patients with hematological diseases, although some may also work at the hematology laboratory viewing blood films and bone marrow slides under the microscope, interpreting various hematological test results and blood clotting test results. In some institutions, hematologists also manage the hematology laboratory. Physicians who work in hematology laboratories, and most commonly manage them, are pathologists specialised in the diagnosis of hematological diseases, referred toas **hematopathologists** or **haematopathologists**. Hematologists and hematopathologists generally work in conjunction to formulate a diagnosis and deliver the most appropriate therapy if needed. Hematology is a distinct subspecialty of internal medicine, separate from but overlapping with the subspecialty of medical oncology.

Hematologists may specialise further or have special interests, for example, in:

- treating bleeding disorders such as hemophilia and idiopathic thrombocytopenic purpura
- treating hematological malignancies such as lymphoma and leukemia (cancers)
- treating hemoglobinopathies
- the science of blood transfusion and the work of a blood bank
- bone marrow and stem cell transplantation

3.2 Serum Biochemistry

Serum biochemistry on the other hand refers to the chemical analysis of blood serum. A profile of tests can be combined to evaluate renal function, electrolyte metabolism, serum proteins, digestion, injury, lipids, pancreatic function, and the liver. Specialised chemistry testing can also be undertaken to assess thyroid glands and liver function. In veterinary clinical pathology, serum biochemistry testing presents the challenges of species differences and low volume samples. Automated analysers for veterinary use have been implemented to provide a precision to sample analysis. Two of these types

of analysers are important tools used in the laboratory of the Division of Comparative Pathology. Preset biochemistry panels are available for use and custom panels can be tailored for the best use in your research project.

Available blood tests with their specificity in mammalian species include:

- Renal function – blood urea nitrogen (BUN) and creatinine
- Electrolytes – chloride, potassium, phosphorus, calcium, magnesium
- Liver – alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, gamma-glutamyltransferase (GGT), total and direct bilirubin, albumin
- Pancreas – amylase, lipase, glucose
- Serum proteins – total protein, albumin, globulin; alpha, beta and gamma globulins via protein electrophoresis
- Injury – creatine kinase (CK), lactate dehydrogenase (LDH)
- Lipids – cholesterol, triglycerides, high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL)

4.0 CONCLUSION

Hematology, also spelled haematology, is the branch of medicine concerned with the study of the cause, prognosis, treatment, and prevention of diseases related to blood. Serum biochemistry on the other hand refers to the chemical analysis of blood serum. A profile of tests can be combined to evaluate renal function, electrolyte metabolism, serum proteins, digestion, injury, lipids, pancreatic function, and the liver. Specialised chemistry testing can also be undertaken to assess thyroid glands and liver function.

5.0 SUMMARY

Hematology, is concerned with the study of the cause, prognosis, treatment, and prevention of diseases related to blood. Hematologists and hematopathologists generally work in conjunction to formulate a diagnosis and deliver the most appropriate therapy if needed. Hematology is a distinct subspecialty of internal medicine, separate from but overlapping with the subspecialty of medical oncology. Serum biochemistry refers to the chemical analysis of blood serum.

6.0 TUTOR-MARKED ASSIGNMENT

1. Briefly explain the term “Hematology”.
2. Briefly explain the term “Serum biochemistry”.
3. State at least five areas of specialisation in hematology.
4. Outline three clinical issues that can be managed by hematologists.
5. Mention seven available blood related tests that have their specificity in mammalian species.

7.0 REFERENCES/FURTHER READING

https://en.wikipedia.org/wiki/Hematology

https://www.acponline.org/about-acp/about-internal medicine/ sub specialties/ hematology

https://en.wikipedia.org/wiki/Hematology

MODULE 4 SAMPLE CONSERVATION AND PESERVATION

UNIT 1 CRYOPRESERVATION

CONTENTS

1.0 INTRODUCTION

Cryo-preservation or **cryo-conservation** is a process in which especially animal and plant material samples such as organelles, cells, tissues, extracellular matrix, organs, seeds or any other biological constructs susceptible to damage caused by unregulated chemical kinetics are preserved by cooling to very low temperatures (typically −80 °C using solid carbon dioxide or −196 °C using liquid nitrogen). At low enough temperatures, any enzymatic or chemical activity which might cause damage to the biological material in question is effectively stopped. Cryopreservation methods seek to reach low temperatures without causing additional damage caused by the formation of ice crystals during freezing. Traditional cryopreservation has relied on coating the material to be frozen with a class of molecules termed cryoprotectants. New methods are constantly being investigated due to the inherent toxicity of many cryoprotectants.By default it should be considered that cryopreservation alters or compromises the structure and function of cells unless it is proven otherwise for a particular cell population. Cryoconservation of animal genetic resources is the process in which animal genetic material is collected and stored with the intention of conservation of the breed.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- know what cryo-preservation is all about
- know different animal sample materials that can be preserved using cryopreservation.

3.0 MAIN CONTENT

3.1 Cryopreservation

Cryo-preservation is a process in which especially animal and plant material samples such as organelles, cells, tissues, extracellular matrix, organs, seeds or any other biological constructs susceptible to damage caused by unregulated chemical kinetics are preserved by cooling to very low temperatures (typically −80 °C using solid carbon dioxide or −196 °C using liquid nitrogen).

3.1.1 Naturalcryopreservation

Water-bears (*Tardigrada*), microscopic multicellular organisms, can survive freezing by replacing most of their internal water with the sugar trehalose, preventing it from crystallisation that otherwise damages cell membranes. Mixtures of solutes can achieve similar effects. Some solutes, including salts, have the disadvantage that they may be toxic at intense concentrations. In addition to the water-bear, wood frogs can tolerate the freezing of their blood and other tissues. Urea is accumulated in tissues in preparation for overwintering, and liver glycogen is converted in large quantities to glucose in response to internal ice formation. Both urea and glucose act as "cryoprotectants" to limit the amount of ice that forms and to reduce osmotic shrinkage of cells. Frogs can survive many freeze/thaw events during winter if no more than about 65% of the total body water freezes.

3.1.1.1 Organisms that Exhibit Natural Cryopreservation

Freeze tolerance, in which organisms survive the winter by freezing solid and ceasing life functions, is known in a few vertebrates which include;

i. Five species of frogs (*Rana sylvatica*, *Pseudacristriseriata*, *Hyla crucifer*, *Hyla versicolor*, *Hylachrysoscelis*), In the case of *Rana sylvatica* one cryopreservant is ordinary glucose, which increases in concentration by approximately 19 mmol/l when the frogs are cooled slowly.
ii. One of salamanders (*Hynobius keyserlingi*),
iii. One of snakes (*Thamnophis sirtalis*)
iv. Three of turtles (*Chrysemyspicta*, *Terrapenecarolina*, *Terrapeneornata*). Snappin g turtles *Chelydraserpentina* and
v. Wall lizards *Podarcismuralis* also survive nominal freezing but it has not been established to be adaptive for overwintering.

3.1.2 History of Cryopreservation

- One of the most important early theoreticians of cryopreservation was James Lovelock.
- In 1953, he suggested that damage to red blood cells during freezing was due to osmoticstress, and that increasing the salt concentration in a dehydrating cell might damage it.
- In the mid-1950s, he experimented with the cryopreservation of rodents, determining that hamsters could be frozen with 60% of the water in the brain crystallised into ice with no adverse effects; other organs were shown to be susceptible to damage.
- This work led other scientists to attempt the short-term freezing of rats by 1955, which were fully active four to seven days after being revived.
- Fowl sperm was cryopreserved in 1957 by a team of scientists in the UK directed by Christopher Polge.
- During 1963, Peter Mazur, at Oak Ridge National Laboratory in the U.S., demonstrated that lethal intracellular freezing could be avoided if cooling was slow enough to permit sufficient water to leave the cell during progressive freezing of the extracellular fluid.
- That rate differs between cells of differing size and water permeability: a typical cooling rate around 1 °C/minute is appropriate for many mammalian cells after treatment with cryoprotectants such as glycerol or dimethyl sulphoxide, but the rate is not a universal optimum.

3.1.3 Temperature

- Storage at very low temperatures is presumed to provide an indefinite longevity to cells, although the actual effective life is rather difficult to prove.
- Researchers experimenting with dried seeds found that there was noticeable variability of deterioration when samples were kept at different temperatures – even ultra-cold temperatures.
- Temperatures less than the glass transition point (Tg) of polyol's water solutions, around −136 °C (137 K; −213 °F), seem to be accepted as the range where biological activity very substantially slows, and −196 °C (77 K; −321 °F), the boiling point of liquid nitrogen, is the preferred temperature for storing important specimens.
- While refrigerators, freezers and extra-cold freezers are used for many items, generally the ultra-cold of liquid nitrogen is required for successful preservation of the more complex biological structures to virtually stop all biological activity.

3.1.4 Risks of Cryopreservation

Phenomena which can cause damage to cells during cryopreservation mainly occur during the freezing stage, and include:

i. **Solution effects:**As ice crystals grow in freezing water, solutes are excluded, causing them to become concentrated in the remaining liquid water. High concentrations of some solutes can be very damaging.
ii. **Extracellular ice formation:**When tissues are cooled slowly, water migrates out of cells and ice forms in the extracellular space. Too much extracellular ice can cause mechanical damage to the cell membrane due to crushing.
iii. **Dehydration**: Migration of water, causing extracellular ice formation, can also cause cellular dehydration. The associated stresses on the cell can cause damage directly.
iv. **Intracellular ice formation:** While some organisms and tissues can tolerate some extracellular ice, any appreciable intracellular ice is almost always fatal to cells.

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Many of these effects can be reduced by cryoprotectants. Once the preserved material has become frozen, it is relatively safe from further damage. However, estimates based on the accumulation of radiation-induced DNA damage during cryonic storage have suggested a maximum storage period of 1000 years.

3.1.4.1 Main Methods to Prevent Risks

The main techniques to prevent cryopreservation damages are a well-established combination of *controlled rate and slow freezing* and a newer flash-freezing process known as *vitrification*.

3.1.5 Vitrification

- Researchers Greg Fahy and William F. Rall helped to introduce vitrification to reproductive cryopreservation in the mid-1980s.
- As of 2000, researchers claim vitrification provides the benefits of cryopreservation without damage due to ice crystal formation.
- The situation became more complex with the development of tissue engineering as both cells and biomaterials need to remain ice-free to preserve high cell viability and functions, integrity of constructs and structure of biomaterials.
- For clinical cryopreservation, vitrification usually requires the addition of cryoprotectants prior to cooling. The cryoprotectants act like antifreeze: they decrease the freezing temperature. They also increase the viscosity.

- Instead of crystallizing, the syrupy solution becomes an amorphous ice—it *vitrifies*. Rather than a phase change from liquid to solid by crystallization, the amorphous state is like a "solid liquid", and the transformation is over a small temperature range described as the "glass transition" temperature.
- Vitrification of water is promoted by rapid cooling, and can be achieved without cryoprotectants by an extremely rapid decrease of temperature (megakelvins per second). The rate that is required to attain glassy state in pure water was considered to be impossible until 2005.

3.1.5.1 Conditions Required for Vitrification

- Two conditions usually required to allow vitrification are an increase of the viscosity and a decrease of the freezing temperature.
- Many solutes do both, but larger molecules generally have a larger effect, particularly on viscosity.
- Rapid cooling also promotes vitrification.
- For established methods of cryopreservation, the solute must penetrate the cell membrane in order to achieve increased viscosity and decrease freezing temperature inside the cell.
- One of the difficult compromises of vitrifying cryopreservation concerns limiting the damage produced by the cryoprotectant itself due to cryoprotectant toxicity.

3.1.6 Areas In Which Cryopreservation is Possible

i. Freezable tissues

- Generally, cryopreservation is easier for thin samples and small clumps of individual cells, because these can be cooled more quickly and so require lesser doses of toxic cryoprotectants.

- Nevertheless, suitable combinations of cryoprotectants and regimes of cooling and rinsing during warming often allow the successful cryopreservation of biological materials, particularly cell suspensions or thin tissue samples.

- Examples include:Semen in semen cryopreservation

ii. **Ovarian tissue in ovarian tissue cryopreservation**

- Plant seeds or shoots may be cryopreserved for conservation purposes.

- Countless cryopreserved cells, vaccines, tissue and other biological samples have been thawed and used successfully.

iii. Preservation of microbiology cultures

- Bacteria and fungi can be kept short-term (months to about a year, depending) refrigerated, however, cell division and metabolism is not completely arrested and thus is not an optimal option for long-term storage (years) or to preserve cultures genetically or phenotypically, as cell divisions can lead to mutations or sub-culturing can cause phenotypic changes.
- A preferred option, species-dependent, is cryopreservation. Nematode worms are the only multicellular eukaryotes that have been shown to survive cryopreservation.

iv. **Fungi**

- Fungi, notably zygomycetes, ascomycetes and higher basidiomycetes, regardless of sporulation, are able to be stored in liquid nitrogen or deep-frozen.
- Crypreservation is a hallmark method for fungi that do not sporulate (otherwise other preservation methods for spores can be used at lower costs and ease), sporulate but have delicate spores (large or freeze-dry sensitive), are pathogenic (dangerous to keep metabolically active fungus) or are to be used for genetic stocks (ideally to have identical composition as the original deposit).
- As with many other organisms, cryoprotectants like DMSO or glycerol (e.g. filamentous fungi 10% glycerol or yeast 20% glycerol) are used.
- Differences between choosing cryoprotectants are species (or class) dependent, but generally for fungi penetrating cryoprotectants like DMSO, glycerol or polyethylene glycol are most effective (other non-penetrating ones include sugars mannitol, sorbitol, dextran, etc.). Freeze-thaw repetition is not recommended as it can decrease viability.
- Back-up deep-freezers or liquid nitrogen storage sites are recommended. Multiple protocols for freezing are summarized below (each uses screw-cap polypropylene cryotubes).

v. Bacteria

- Many common culturable laboratory strains are deep-frozen to preserve genetically and phenotypically stable, long-term stocks. Sub-culturing and prolonged refrigerated samples may lead to loss of plasmid(s) or mutations.

- Common final glycerol percentages are 15, 20 and 25. From a fresh culture plate, one single colony of interest is chosen and liquid culture is made.
- From the liquid culture, the medium is directly mixed with equal amount of glycerol; the colony should be checked for any defects like mutations.
- All antibiotics should be washed from the culture before long-term storage. Methods vary, but mixing can be done gently by inversion or rapidly by vortex and cooling can vary by either placing the cryotube directly at −50 to −95 °C, shock-freezing in liquid nitrogen or gradually cooling and then storing at −80 °C or cooler (liquid nitrogen or liquid nitrogen vapor).
- Recovery of bacteria can also vary, namely if beads are stored within the tube then the few beads can be used to plate or the frozen stock can be scraped with a loop and then plated, however, since only little stock is needed the entire tube should never be completely thawed and repeated freeze-thaw should be avoided. 100% recovery is not feasible regardless of methodology.

vi. Worms

The microscopic soil-dwelling nematode roundworms Panagrolaimusdetritophagus and Plectusparvus are the only eukaryotic organisms that have been proven to be viable after long-term cryopreservation to date. In this case, the preservation was natural rather than artificial, due to permafrost.

vii. Embryos
viii. Ovarian tissue
ix. Semen
x. Testicular tissue
xi. Moss

4.0 CONCLUSION

Cryopreservation is storage at ultra-low temperature (–196ºC), usually in liquid nitrogen. All cellular division and metabolic processes stop at this temperature, so plant material can be stored without alteration or modification for an unlimited period of time in a small volume, protected from contamination and requiring very little maintenance. Cryopreservation currently offers the only safe and cost-effective option for the long-term conservation of genetic resources of vegetatively propagated species.

5.0 SUMMARY

Cryopreservation methods seek to reach low temperatures without causing additional damage caused by the formation of ice crystals during freezing. Traditional cryopreservation has relied on coating the material to be frozen with a class of molecules termed cryoprotectants. New methods are constantly being investigated due to the inherent toxicity of many cryoprotectants.

6.0 TUTOR-MARKED ASSIGNMENT

1. Discuss exhaustively, the concept of cryopreservation or cryoconservation.
2. What is natural cryopreservation? state five organisms which exhibit natural cryopreservation?
3. Briefly outline the history of cryopreservation.
4. Explain the role of temperature in cryopreservation.
5. State and briefly explain the risks of cryopreservation.
6. What do you understand by vitrification andwhat are the conditions required by vitrification?
7. Mention and briefly discuss areas where cryopreservation is possible.

7.0 REFERENCES/FURTHER READING

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