SAC 454 Instrumental Methods of Analysis (1+1)

SYLLABUS

Objective

To familiarize students with the design, operational principles and practical applications of modern instruments used for the quantitative analysis of soil, plant water, manure and fertilizer samples

Unit I

Principles of instrumentation- classification of instrumental methods – selection of instruments - Principles involved in digestion methods – dry ashing and wet digestion – open vs closed digestion - Block digester, microwave digester and IR digestion systems - components -operation - special consideration

Unit II

Automated methods – Principle and applications - Total N analyser, CN analyser

Unit III

Optical methods – spectrophotometry – visible, ultraviolet and infrared spectrometry - Principle - Instrumentation – sample handling and measurement - method development and validation - accuracy

Unit IV

Emission and absorption Spectroscopy - principles and applications - flame photometry, atomic absorption spectrophotometer, inductively coupled plasma emission spectrometry - instrumentation - features and operation of components - sample handling - errors - fault finding - trouble shooting

Unit V

Chromatography techniques – classification - paper chromatography, TLC - Gas chromatography- HPLC, GC – MS - principles - Instrumentation - sample preparation and handling - errors – trouble shooting

THEORY SCHEDULE

1. Basic principles in instrumental method of analysis
2. Principle and practice of digestion methods
3. Principle and practice of N analyser and CN analyser
4. Spectrophotometry: Types, Principle and instrumentation
5. Spectrophotometry: Sample handling and measurement, method development and validation and checking for accuracy
6. Emission spectroscopy (Flame Photometer): Instrumentation, interferences, trouble shooting and maintenance
7. Absorption spectroscopy (Atomic Absorption Spectrophotometer): Principle, instrumentation, features and operation of components

SAC 454

8. Absorption spectroscopy (Atomic Absorption Spectrophotometer): Sample handling and measurement, errors due to molecular and ionic species, matrix effect and other interferences, trouble shooting and maintenance
9. Mid semester examination
10. Absorption spectroscopy (Inductively Coupled Plasma Emission Spectrometer): Concepts and instrumentation
11. Absorption spectroscopy (Inductively Coupled Plasma Emission Spectrometer): Preparation of samples and standards, interferences, trouble shooting and maintenance
12. Principle and practice of paper chromatography, Thin layer chromatography
13. Gas chromatography: Principle, Types and instrumentation
14. Gas chromatography: Operation, sample handling, maintenance and trouble shooting and applications
15. High Performance Liquid Chromatography: Principle, Instrumentation and operation
16. High Performance Liquid Chromatography: Sample preparation, method development, maintenance and troubleshooting
17. GC – MS: Principle, instrumentation, Sample preparation, method development, maintenance and troubleshooting

PRACTICAL

Collection and processing of samples - Digestion of samples – block digester and microwave digester methods - N analyser – Spectrophotometer - UV –Vis Spectrophotometer -- Emission spectroscopy - Flame photometer – Absorption spectroscopy – Atomic Absorption Spectrophotometer (AAS) and Inductively Coupled Plasma Emission Spectrometer (ICP) – Chromatography - Gas Chromatography and High Performance Liquid Chromatography- Procedures for establishing a analytical laboratory

PRACTICAL SCHEDULE

1. Collection and processing of samples (Soil, plant, water, manure and fertilizer)
2. Digestion of samples by block digester/microwave digester and sample preparation for different analysis
3. N anlyser: Calibration, sample estimation and results interpretation
4. UV-Vis spectrophotometer: Getting acquainted with parts of UV -Vis spectrophotometer and preparation of standards
5. UV-Vis spectrophotometer: Calibration, sample estimation (P/S/B) and results interpretation
6. Flame photometer: Getting acquainted with components offlame photometer and preparation of standards, calibration, sample estimation (P/S/B) and results interpretation

SAC 454

7. Getting acquainted with components of AAS and standards (micronutrients and heavy metal) preparation
8. Calibration and sample estimation for micronutrients and heavy metals by AAS
9. Getting acquainted with components of ICP
10. Calibration, sample estimation for elements by ICP and results interpretation
11. Analyte extraction from sample and separation for GC
12. Calibration, sample introduction and interpretation of results in GC
13. Analyte extraction from sample and separation for HPLC
14. Calibration, sample introduction and interpretation of results in HPLC
15. Visit to a analytical laboratory (Government/Private)
16. Procedures for establishing a analytical laboratory – Guidelines and budget
17. Practical examination

Outcome: At the end of the course the student will be able to handle sophisticated instruments. The knowledge earned will help in establishing a analytical laboratory for analyzing soil/plant/water/fertilizer/manure samples.

References

Chatwal, G. and Anand. 2005. *Instrumental Methods of Chemical Analysis*. Academic Press.

MuralidharaRao, D., A.V.N Swamy and D.Dharaneeswara Reddy.2013. *Instrumental Methods of Analysis*. CBS Publishers & Distributors Pvt.Ltd., New Delhi.

Skoog, A., Holler, F and Nieman, A. 2007. *Principles of Instrumental Analysis* (6th Ed.). Thomson Brooks/Cole publishing. ISBN: 9780030020780.

Willard M, Merritt LL & Dean JA.1989. *Instrumental Methods of Analysis*. (7th Ed). Wadsworth Publishing Compnay, Boston, USA. ISBN :9780534001423.

e resources

http://blogs.rediff.com/ticconcvibi1985/2013/05/12/download-ebook-modern-hplc-for-practicing-scientists-free/

http://cdn.intechopen.com/pdfs-wm/26275.pdf

http://faculty.ksu.edu.sa/Dr.almajed/Books/practical%20HPLC.pdf

http://web.uni-plovdiv.bg/plamenpenchev/mag/books/spectroscopy/PRIMER.PDF

SAC 454

LECTURE 1

BASIC PRINCIPLES IN INSTRUMENTAL METHOD OF ANALYSIS

Introduction

Analytical chemistry is a branch of chemistry that deals with the characterization of chemical composition of materials of in terms of elements or compounds present in them.

"Father of Analytical Chemistry" is **IzaakMauritsKolthoff**

General principles of analytical chemistry

Analytical chemistry is based on the changes in physical and chemical properties of a substance.

Physical changes lead to alteration of properties of a substance, without actually affecting the substance. Eg. Hammering a metal modifies the shape or increases hardness; freezing or boiling of water. Such properties aid in separation or purification.

Chemical changes lead to change in substance, and entirely a new substance is formed in the process due to changes like combination, decomposition, replacement and double decomposition or a combination of these processes.

E.g. Combination: Direct joining of two or more simple substances to form a more complex compound

$2Cu+O_2$ ---------- $2CuO$

All chemical changes involve either one or a combination of these processes.

Types of analytical chemistry

There are two types

I. Qualitative II. Quantitative

I. Qualitative analytical chemistry

Qualitative analysis deals with the identification of chemical species present in the sample.

Different methods of qualitative analysis

1. Microcrystalloscopic analysis

For detecting cations and anions, use of reactions which result in the formation of compounds of characteristic crystal shape. The form and rate of crystallization are influenced by the conditions of a reaction

2. Pyrochemical analysis

On heating substances in a flamedifferent characteristic phenomena like sublimation, evaporation, melting, colour change and flame coloration may be observed.

- **Flame coloration**
  When a salt is sprinkled on flame, appearance of green colour shows the presence of iron, copper by blue colour and calcium by brick red colour.
- **Sublimation**
  It is the process of conversion of solid to vapour without becoming liquid: Eg. sulphur, selenium, iodine and chlorides of mercury (II), antimony (III) and (V), bismuth (III) and zinc.

3. Spot tests

It refers to micro and semi micro test involving only few drops of test solution and a reagent. It may be carried out by any one of the following processes.

i. A drop of test solution and a drop of the reagent may be brought together on a glass or porcelain surface.
ii. A drop of test solution may be placed on a filter paper strip impregnated with reagents
iii. A drop of reagent may be subjected to the action of gas liberated from a drop of test solution

Eg. Profuse effervescence by adding dilute HCl to the carbonate containing material

4. Analyzing by grinding powders

Coloured reaction products are obtained when some substances are mechanically ground with reagents. Mechanical grinding helps to increase the surface area thereby increase the rate of chemical reactions. Eg. Murexide

5. Luminescence analysis

Many substances are capable of glowing under the action of different types of energy. All types of light emission, excluding the glow of heated bodies, are called luminescence. This type of analysis is used in identification of substances with smaller concentrations and to determine the purity of a substance.

II. Quantitative analytical chemistry

Quantitative analysis is used to quantify the compounds and to determine ratios of the constituents in a substance.

In general, quantitative analysis is grouped into Ultimate, Proximate, Complete and Partial Analysis.

a. Based on nature of constituents to be analyzed

- **Ultimate Analysis**

An analysis which permits the determination of the chemical elements of unknown compound is referred as an "ultimate" analysis. Eg. C, H, O, N, P, K Ca, Mg, Na etc

- **Proximate Analysis**

An analysis which is used to determine the quantities of radicals, compounds, or classes of compounds. Eg.Estimation of Protein, fat, Carbohydrates, sugars, starches etc.

b. Based on number of constituents to be analyzed

- **Complete Analysis**

Complete analysis is one in which all the constituents in the substance are analyzed.

Eg. Estimation of Na and Cl ions in NaCl

- **Partial Analysis**

Partial analysis deals with the determination of one or two constituents / specific constituents in a sample.Eg. Estimation of Na alone in NaCl

c. Based on quantity of sample required for analysis

a. Macro analysis : > 0.1 g
b. Semimicro analysis : 0.01 – 0.1 g

c. Micro analysis : 0.001 – 0.01 g

d. Sub micro / Ultramicro analysis : < 1 mg

General classification of quantitative analytical methods

I. Conventional or classical methods

a) Volumetric analysis: Volume of standard reagent solution reacting with the analyte

(i). Neutralisation reaction - Reaction between acids and bases

(ii). Redox reaction - Reaction between oxidative and reductive reagents

(iii). Precipitation reaction - Formation of insoluble precipitates

(iv).Complex formation - Reacting constituents form undissociable stable complexes

b) Gravimetric analysis: Weight of pure analyte or stoichiometric compound containing in it.

c) Gas analysis: Quantification of gas released from biological system

II. INSTRUMENTAL METHODS

1. Electrical methods

a) Potentiometry - Measurement of electrode potential in equilibrium with an ion to be determined

b) Conductometry - Measurement of electrical conductivity or resistance of a substance

c) Voltametry - Measurement of microelectrode potential at a specific voltage

d) Coulometry - Measurement of current and time required for a chemicalchange

2. Optical /Spectral methods

a) Absorption

i) **Visible spectrum** - Absorption of light by a coloured constituent of an analyte at visible range - (Colorimeter-wavelength isolation by filter)

ii) **UV - VIS spectrum** - Absorption of light by a coloured constituent of an analyte at visible as well as UV range (Spectrophotometer - wavelength isolation by prism or grating)

iii) **IR spectrum**- Molecular structure of the sample is analysed by measuring the absorbance spectrum of a sample at IR wavelength

b) Emission

i. Emission spectroscopy- Measurement of intensity of radiation emitted by atoms that are excited by light /electrical /thermal energy

ii. Flame photometry- Measurement of intensity of radiation emitted by atoms that are excited by thermal energy

iii. Flourimetry- Measurement of fluorescence light emitted by excitation of substance with a beam of UV light

c). Others

a) **Separation methods** - Separation of components of a mixture due to the difference in density, adsorption and mobility(Essential oil extraction, Thin Layer Chromatography)

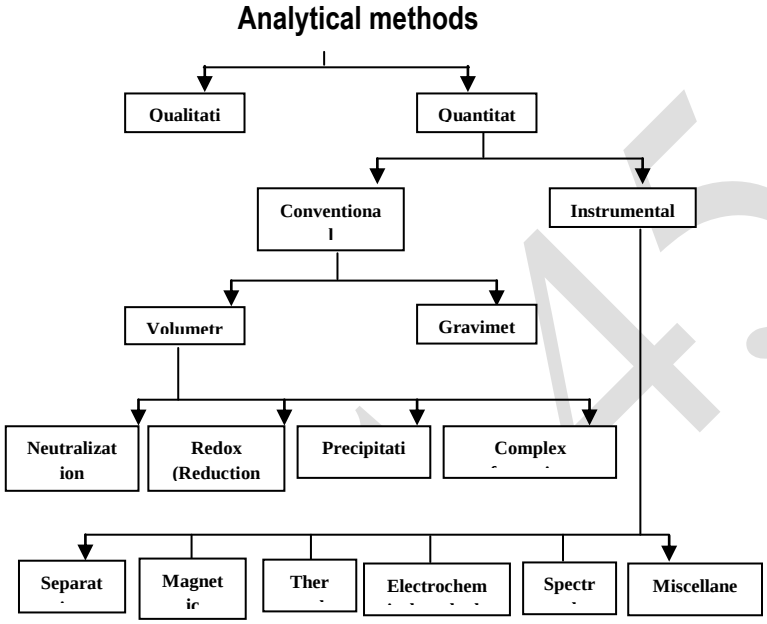
b) **Magnetic methods**-Absorption and reemission of electromagnetic radiation in the presence of magnetic field (Nuclear Magnetic Resonance spectroscopy)

c) **Thermal methods**-Measurement of temperature variation under identical thermal cycles between reference and test sample over a period of time (Differential Thermal Analyzer)

d) **Radiation methods**- Measurement of intensity of nuclear radiations emitted by the analyte (Geiger Muller Counter)

e) **Massspectroscopic**- Separation of charged gas molecules (ions) according to their mass (Mass spectrometer)

Modern methods of instrumental analysis have evolved from the earlier classical methods which were dependent on measurement of mass or volumes. Such classical methods were time-consuming and were limited by errors both determinate and indeterminate.



Modern instrumental methods came to the aid of the analytical chemist by providing the following distinct benefits:

- **Time saving** – Analysis time has been reduced from several hours or days to even Minutes.
- **Improvement in accuracy** of results by elimination of errors introduced due to personal bias.
- **Improvement in sensitivity** leading to trace analysis at ppt or even femto- mole levels. Such sensitivities could not be imagined using the conventional

classical approach.

- **Fast decisions** in case of online analysis during manufacturing operations or deciding on viability before taking commercial decisions.

Instrumental versus Non-instrumental methods

The dividing line between instrumental and non-instrumental methods is very fine and often fades out. Even the classical methods make use of devices such as analytical balance or volumetric apparatus. It can be said that instrumental techniques involve use of electromechanical gadgets comprising of electronic components and circuits, optical elements and mechanical parts. Analytical chemists have

exploited the properties of materials based on general principles of physics, chemistry, biology, computer science and engineering.

Some common material properties which have been exploited are:

- Electrical conductivity or resistance
- Absorbance or emission of electromagnetic radiation
- Phase transitions of materials due to heat absorption or liberation
- Temperature changes
- Acidity level changes in the medium
- Viscous behaviour changes

The analytical scientist has been able to realize the potential benefits of instrumental analysis mainly in the last 4 to 5 decades. Over the period it has become possible to increase laboratory throughputs, achieve specific detection levels not imagined before, multi component analysis in single sample and trace detection of an analyte in complex sample matrices.

Modern instrumental techniques offer unlimited advantages but one should always bear in mind that basic analytical operational skills cannot be overlooked due to over dependence on instrumental techniques. Such basic skills ensure high reliability of results if required attention and care are exercised at time of sample extraction, treatment and handling during preservation prior to analysis.

Advantages and limitation of instrumental methods of analysis

Advantages of instrumental methods

1. A small amount of a sample is needed for analysis.
2. Determination by instrumental method is considerably fast.
3. Complex mixture can be analysed either with or without their separation.
4. Sufficient reliability and accuracy of results are obtained by instrumental method.
5. When non-instrumental method is not possible, instrumental method is the only answer to the problem.

Limitation of instrumental methods

1. In general, instrumental methods are costly because of cost, maintenance and trained personnel required for their handling.
2. The sensitivity and accuracy depend upon the type of instrument.
3. Specialized training for handling instrument is required.
4. There is frequent need of checking results with other methods.
5. In some cases, instrumental method may not be specific.

Precision Vs Accuracy

Precision is the degree of agreement between the measured value and other values obtained under same condition. It refers to the reproducibility of values. It is determined by the magnitude of random errors.

Precision may be expressed mathematically as the standard deviation, coefficient of variation, range of data or as confidence level (95%) about the mean value. More the number of samples, higher will be the precision.

Accuracy is the degree of agreement between the measured value and the true value. It refers to the reliability of the results. It is determined by the magnitude of determinate errors. Analytical results that are of a high degree of precision are not necessarily of a high degree of accuracy.

Accuracy can be determined by

- **Absolute method:** By using a sample containing known quantity of the constituent
- **Comparative method:** Sample is determined by two independent methods and the accuracy is compared. The permissible error for a sample to be accurate is 1-10 ppt (parts per thousand)

Eg:- An analyst consistently making the same mistake (Eg. wrong reagent) and though his results are precise, they are not accurate.

i. When A,B, and C determine the weight of a substance, if A records the weight as 18.0 g, B as 18.2 g and C as 19.5 g and the actual weight is 20g, then **C is accurate** while **A and B are precise**.

Errors In Estimations

Error refers to the numerical difference between measured value and the true value

1. Absolute Error (E): This is the difference between observed or measured value (O) and the true value (T) of the quantity measured.

Absolute error = True value (T) – Observed value (O)

Eg. Estimated nitrogen content in urea is 45.5 % while the true value is 46 %

Absolute error (E) = 46.0 – 45.5 = 0.5 %

2. Relative error (R): It is the ratio of the absolute error and the true value. It is expressed as % or parts per thousand

$$\text{Relative error} = \frac{\text{Absolute error}}{\text{True value}} \times 100$$

Eg. Estimated nitrogen content in urea is 45.5 % while the true value is 46 %

Relative error = (0.5 / 46.0) x 100 = 1.08 %

3. Determinate/ Constant errors / Systematic errors: Errors that can be determined and eliminated or reduced to the minimum by following certain techniques. They are grouped as

a. Physical errors – Loss of material at various stages of analysis i.e., while washing, filtration or transfer.
b. Personnel errors- It is due to the inability of the analyst to fix the end point due to colour blindness or lack of knowledge in analysis
c. Errors in methods - Errors which occur due to incorrect method of sampling, wrong choice of a reaction and incompleteness of the reactions
d. Instrumental errors – Errors which occur due to the environmental factors acting on the instrument
e. Additive errors – It is the error added to the true value and does not depend on the true value itself. Eg. Loss in weight of a crucible in which a precipitate is ignited.

f. Proportional errors – A proportional error is the error which depends on the true value. Eg. Impurities present in a standard compound.

4. **Indeterminate errors**- The errors which are unknown but occur in the analysis accidentally. The source of these errors cannot be determined. They are also called random/accidental errors.

   Eg. Drift in electronic circuit

5. **Gross / Crude errors –** These are errors which seriously alter the analytical results. Eg. Use of numerically incorrect conversion factors.

CALIBRATION OR STANDARDIZATION

- Calibration or Standardization is the process of establishing **the response of a detection** or measurement system **to known amounts**/ concentrations of an analyte.
- With the exception of absolute methods of analysis that involve chemical reactions of known stoichiometry (e.g., gravimetric and titrimetric determinations), a calibration or standardization procedure is required to establish the relation between a measured **physico-chemical response** to an analyte and the **amount or concentration** of the analyte producing the response.
- Techniques and methods where calibration is necessary are frequently instrumental, and the detector response is in the form of an electrical signal.
- An important consideration is the effect of matrix components on the analyte detector signal, which may be suppressed or enhanced**, this being known as the matrix effect.**
- When this is known to occur, **matrix matching of the calibration** standards to simulate the gross composition expected in the samples is essential (i.e. matrix components are added to all the analyte standards in the same amounts as are expected in the samples).

Methods of calibration

Choice of methods depends on

- analytical technique to be employed, the nature of the sample and the level of analyte(s).

These include:

- External standardization
- Internal Standardization

External standardization

- Carried out by a series of at least four calibration standards containing known amounts or concentrations of the analyte and matrix components.
- Response of the detection system is recorded for each standard under specified and stable conditions and additionally for a blank, sometimes called a reagent blank (a standard prepared omitting the analyte).
- Data is either plotted as a calibration graph or used to calculate a factor.

Standard addition

This is a calibration procedure that avoids matrix interference by

- Measuring instrument response for an analyte in both the sample and a sample to which known amounts of an analyte standard have been added.
- Where components of a sample other than the analyte(s) (the matrix) interfere with the instrument response for the analyte, the use of a calibration curve based on standards of pure analyte may lead to erroneous results.
- Such matrix interference effects can be largely if not entirely avoided by preparing calibration standards where known amounts of pure analyte are added to a series of equal sized portions of the sample, a procedure known as spiking.

Internal standardization

This is a calibration procedure where the ratio of the instrument response for an analyte to that of an added standard is measured for a series of analyte standards and samples. For some analytical techniques, particularly chromatography, variations in experimental conditions can adversely affect the precision of the data.

A calibration procedure whereby a constant amount of a selected substance, the internal standard is added to all samples and analyte standards alike compensates for variations in sample size and other parameters.

The ratio of the detector response for the analyte in each standard to the corresponding response for the added internal standard is plotted on the y axis of a calibration graph against the mass or concentration of the analyte on the x-axis.

The correlation coefficient, slope and intercept values can be computed

- Instruments and apparatus used for analytical work must be correctly maintained and calibrated against reference values to ensure that measurements are accurate and reliable. Performance should be checked regularly and records kept so that any deterioration can be quickly detected and remedied.

Some examples of instrument or apparatus calibration are

● Manual calibration of an electronic balance with certified weights;

● Calibration of volumetric glassware by weighing volumes of pure water;

● Calibration of the wavelength and absorbance scales of spectrophotometers with certified emission or absorption characteristics;

● Calibration of temperature scales and electrical voltage or current readouts with certified measurement equipment.

SAC 454

LECTURE 2

PRINCIPLES AND PRACTICE OF DIGESTION METHODS

Preparation of samples

After collection of a sample, solution of the analyte must be prepared for analysis. This is achieved by drying, extraction, concentration or precipitation depending upon the analyte.

i. Drying

Soil samples contain variable amounts of adsorbed water. The samples are placed in a drying oven at 105 to 110^{o}C for 8 hours. Plant samples can be shade dried and later oven dried at a temperature of 60-70^{o}C for 8 hours.

ii. Dissolution of samples

Dissolution of samples for organic and inorganic analytes varies widely. Sample preparation is of totally destructive for inorganic analyteeg.,Kjeldahl analysis, in which organic nitrogen is converted to ammonium ion. For organic analytes, the non-destructive / partially destructive method is used.

B. Digestion Techniques

Digestion is the process in which the mineral constituents are brought into solution. In plant sample preparation, especially digestion procedure is carried out with the aim of separating analyte from the matrix, and to avoid interaction of organic substance with metal ions or chemical reagents.

The choice of digestion procedure depends on the

- nature of organic substance and the content of inorganic components
- choice of heavy metal for analysis, or the method that we are going to use for determination.

Types of digestion techniques.

I. Ashing

(i)Dry ashing procedures use a high temperature muffle furnace capable of maintaining temperatures of between 200 and 600 °C. Water and other volatile materials are vaporized and organic substances are burned in the presence of oxygen in air to CO2, H2O and N2. Most minerals are converted to oxides, sulfates, phosphates, chlorides or silicates (Tuzenet al., 2004).

The sample is placed in a suitable crucible (silica, platinum or pyrex glass) and ignited in a muffle furnace at a temperature of 500 - 550^{o}C to obtain an inorganic residue / white ash. It is the best technique for samples that contain more organic matter and are to be analysed for non volatile substances. It is used in the estimation of nutrients like Fe, K, Ca, Mg and Mn which are present in substantial quantities.

Advantages

- safe,

- Simple and free from contamination as no reagents or few reagents are required,
- many samples can be analyzed simultaneously and less labour
- Dry ashing provides good precision and is an easy,
- rapid digestion method requiring minimal analyst attention.
- An additional benefit is that this method is relatively free from reagent contamination

Demerits

- Loss of some elements by volatilization
- Contamination of the sample by airborne dust
- Irreversible sorption of the analyte into the walls of the vessel.

Types of dry ashing

a. Low Temperature Ashing

Activated oxygen radicals are very reactive and attack organic matter at low temperatures. Temperatures of less than 100^{o}C can be maintained and volatilization losses are minimized.

b. Oxygen combustion

Elemental analysis in organic compounds for carbon or hydrogen is performed by oxygen combustion in which carbon is converted to CO_2 and hydrogen to H_2O which is absorbed by suitable absorbents like **sodium hydroxide or asbestos (Ascarite) for CO_2** and **magnesium perchlorate (Dehydrite) for H_2O**. The gain in weight of the absorption tubes containing absorbents is a measure of CO_2 and H_2O liberated from the sample.

(ii) Sulphatedashing

(iii) Wet ashing

II.Wet digestion

In wet digestion, the acids oxidize organic matter to carbon di oxide, water and other volatile products leaving behind salts and the inorganic constituents. Wet digestion with a mixture of nitric, sulphuric and perchloric acids is commonly preferred. The mixture is more efficient in the presence of Molybdenum (VI) catalyst and digestion is usually performed in Kjeldahl flask. Nitric acid destroys the organic matter. Digestion is continued until clear white solution is obtained.

Merits

- Rapidity
- Low level of temperature is maintained
- No loss by retention

Demerits

- Introduction of impurities from the reagents

SAC 454

Types of wet digestion

a. Open flask digestion

If more of silica is present, the sample is digested thoroughly with nitric acid in a Poly tetra fluro ethylene (PTFE) beaker followed by addition of perchloric and hydrofluoric acids. Aqua regia (3:1 mixture of conc. HCl and HNO_3) dissolves noble metals. Volatile products are lost in this method.

Eg. Determination of nitrogen containing compounds by Kjeldahl digestionin the presence of digestion mixture (potassium sulphate: copper sulphate in 10:1)

b. Pressurized sealed container digestion

It is applicable for acid digestion of samples. The weighed samples are placed in small quartz vessels with appropriate acid digestion solution, sealed with PTFE and quartz caps are placed in a heating block. The apparatus is closed and pressurised with nitrogen. No volatilization loss occurs but carbon is not removed.

c. Microwave digestion

Containers in microwave digestion are made of high temperature resistant polymers which do not contain metal contaminants. This method eliminates the chance of airborne contamination, reduces evaporation loss of volatile elements. For ashing samples, temperature of 300^{o}C and pressure of 800 psi is applied. Pressure and temperature can be controlled hence results in reproducible digestion. Digestion is completed within than 30 minutes. Well suited for environmental and biological samples.

Microwave wet digestion in a closed system uses microwave energy to heat the solvent, which in contact with the sample is converted to a solution (Stalović and Đorđević, 2013). Microwaves are non-ionizing electromagnetic radiation. Molecules exposed to microwave radiation undergo molecular motion by the migration of ions and the rotation of dipoles without changing the structure. Microwave energy has the frequency range from 300 to 300,000 MHz.

Merits

- shorter acid digestion time,
- better recovery of volatile elements,
- lower contamination levels,
- minimal volumes of reagents are required,
- more reproducible procedures and a better working environment

Demerits

- Inability to add reagents during digestion.
- Amount of sample that can be used is less (1 g or less)
- Safety concerns due to the use of high pressure and corrosive agents.

Reagents commonly used in digestion / dissolution

SAC 454

Strong mineral acids are good solvents for many inorganics. Hydrochloric acid is a general solvent for dissolving metals. Nitric acid dissolves most of the common metals, non ferrous alloys and acid insoluble sulfides. Perchloric acid must be used with caution as it reacts explosively with many oxidizable substances.

III. FUSION TECHNIQUE

Fusion decomposition provides an alternative digestion method. This is a high-temperature technique where powdered samples are heated with a suitable flux to produce a residue that may be readily dissolved. Fusion decompositions are the most rigorous digestions available and all silicate materials can be brought into a complete solution when fused with an appropriate flux. Inorganic samples that resist digestion with acids or bases can be brought into solution by fusing with large excess of an alkali metal called flux. The sample and flux are mixed together in a crucible and heated till the substance fuse together in a molten state. The melt dissolves readily in distilled water or dilute acids. **Sodium carbonate**and **lithium metaborate ($LiBO_3$)** are the commonly used basic flux. This flux introduces only Li and B into the final solution and is used at a relatively low flux: sample ratio of 3:1.

Disadvantage of the fusion technique:

- The introduction of extra salts into the final solution from the flux (thereby increasing total dissolved solids).
- Contamination from the added flux and the loss of volatile materials is the drawback of this method.

Fusion decomposition remains the preferred technique for quantitative analysis of silicon and may be the only practical method for complete decomposition of refractory minerals such as zircon, rutile, and cassiterite.

Practical Analytical Tips

Digestion of Soil, Water, Fertilizer and Manure Samples

1. **Soil**

   Total nutrients: Na_2CO_3 fusion extract

   Total nitrogen: Digestion with sulphuric acid- salicylic acid mixture in the presence of sodium thiosulphate. **Potassium sulphate – Copper sulphate (10:1) is the digestion mixture**. Copper sulphate acts as a catalyst and indicates the completion of the reaction. Potassium sulphate raises the boiling point during digestion.

   Total phosphorus:60 ml of 1:1 HCl is added and digested over a sand bath for 8 hours

2. **Fertilizer**

Urea: The amide form of nitrogen is converted to ammoniacal nitrogen by digesting with sulphuric acid in the presence of digestion mixture

Total phosphorus: Digesting with conc. HCl and HNO_3

3. **Organic manures**

   Nitrogen: Digestion with sulphuric acid – salicylic acid mixture

   P & K: Digestion with triple acid mixture (HNO_3, H_2SO_4, $HClO_4$ in 9:2:1)

4. **Plant samples**

   Nitrogen: Digestion with diacid mixture (H_2SO_4, $HClO_4$ in 5:2)

   P & K: Digestion with triple acid mixture (HNO_3, H_2SO_4, $HClO_4$ in 9:2:1)

SAC 454

LECTURE 3

PRINCIPLES AND PRACTICE OF N ANALYSER AND CN ANALYSER

Semi automatic N/protein analyser

It is the instrument used to determine the nitrogen from the digested sample wherein digestion should be done externally and N should be brought to solution phase before feeding into N analyser. It is working based on Kjeldahl principle.

The Kjeldahl method or Kjeldahl digestion in analytical chemistry is a method for the quantitative determination of nitrogen contained in organic substances plus the nitrogen contained in the inorganic compounds ammonia and ammonium (NH_3/NH_{4+}). Without modification, other forms of inorganic nitrogen, for instance nitrate, are not included in this measurement. This method was developed by Johan Kjeldahl in 1883.

The method consists of heating a substance with sulphuric acid, which decomposes the organic substance by oxidation to liberate the reduced nitrogen as ammonium sulphate. In this step potassium sulphate is added to increase the boiling point of the medium (from 347 °C to 373 °C). Chemical decomposition of the sample is complete when the initially very dark-coloured medium has become clear and colourless. The solution is then distilled with a small quantity of sodium hydroxide, which converts the ammonium salt to ammonia. The amount of ammonia present, and thus the amount of nitrogen present in the sample, is determined by back titration. The end of the condenser is dipped into a solution of boric acid. The ammonia reacts with the acid and the remainder of the acid is then titrated with a sodium carbonate solution by way of a methyl orange pH indicator.

Degradation: Sample + $H_2SO_4 \rightarrow (NH_4)_2SO_4(aq) + CO_2(g) + SO_2(g) + H_2O(g)$

Liberation of ammonia: $(NH_4)_2SO_4(aq) + 2NaOH \rightarrow Na_2SO_4(aq) + 2H_2O(l) + 2NH_3(g)$

Capture of ammonia: $B(OH)_3 + H_2O + NH_3 \rightarrow NH_4^+ + B(OH)_4^-$

Back-titration: $B(OH)_3 + H_2O + Na_2CO_3 \rightarrow NaHCO_3(aq) + NaB(OH)_4(aq) + CO_2(g) + H_2O$

In practice, this analysis is largely automated; specific catalysts accelerate the decomposition. Originally, the catalyst of choice was mercuric oxide. However, while it was very effective, health concerns resulted in it being replaced by cupric sulfate. Cupric sulfate was not as efficient as mercuric oxide, and yielded lower protein results. It was soon supplemented with titanium dioxide, which is currently the approved catalyst in all of the methods of analysis for protein in the Official Methods and Recommended Practices of AOAC International.

Limitations

Kjeldahl method is not applicable to compounds containing nitrogen in nitro and azo groups and nitrogen present in rings (e.g. pyridine, quinoline, isoquinoline) as nitrogen of these compounds does not convert to ammonium sulphate under the conditions of this method.

N/CH/CHN/CNS element analyser

Elemental combustion analysis of solid materials (soil or rock powder) is based on the transformation of solids into the gas phase by rapid and complete flash combustion of the sample material. The concentrations are then measured by gas chromatography. Mostly these instruments works based on Dumas /Combustion principle.

The Dumas method in analytical chemistry is a method for the quantitative determination of nitrogen in chemical substances based on a method first described by Jean-Baptiste Dumas in 1826. The Dumas technique has been automated and instrumentalized, so that it is capable of rapidly measuring the elements particularly N, C, H and S from different samples.

CN ELEMENTAL ANALYZER

*A **CN/CHN/CNS Analyzer*** is a scientific instrument which can determine the elemental concentrations in a given sample. It is used to measure carbon, hydrogen/sulfur and nitrogen. Sample sizes are most often just a few milligrams, but may differ depending on system. For some sample matrices larger mass is preferred due to sample heterogeneity.This instrument uses combustion to oxidize the sample into simple compounds which are then detected with thermal conductivity detection or infrared spectroscopy. Separation of interference is done by chemical reagents.

Components / zones of CN analyser and their function

It comprised of four major zones

- Combustion Zone
- Gas Control Zone
- Separation Zone

• Detection Zone

(i). Combustion Zone, samples encapsulated in tin are inserted automatically using an auto injector. In the presence of excess oxygen and combustion reagents, the samples are completely combusted. Oxides of nitrogen are reduced to elemental nitrogen, N_2. The flexibility of optimizing static and dynamic combustion conditions to meet the specific sampling need of is given here.

(ii). Gas Control Zone :The product gas then passes through a water trap into the Gas Control Zone and is captured in the mixing chamber. Here, the gas is rapidly mixed for thorough homogenization and precisely maintained at controlled conditions of pressure, temperature and volume, which leads to highly reproducible results.

(iii). Separation Zone:After the homogenization of the product gas, the mixing chamber is depressurized through a column is the Separation Zone of instrument. The separation approach used is a technique known as Frontal Chromatography. As the gas elutes, it is measured by a thermal conductivity detector in the Detection Zone of the analyser.

*The **thermal conductivity detector** (**TCD**),* also known as a **katharometer**, is a bulk property detector and a chemical specific detector commonly used in gas chromatography. This detector senses changes in the thermal conductivity of the column effluent and compares it to a reference flow of carrier gas. Since most compounds have a thermal conductivity much less than that of the common carrier gases of helium or hydrogen, when an analyte elutes from the column the effluent thermal conductivity is reduced, and a detectable signal is produced.

***Infrared spectroscopy** (**IR spectroscopy** or **vibrational spectroscopy**)* involves the interaction of infrared radiation with matter. It covers a range of techniques, mostly based on absorption spectroscopy.

Instrumentation and estimation

Solid samples are weighed in tin containers (tin is of importance for the right combustion in the elemental analyzer) and loaded into an automatic sampler. The tin cups are then dropped in a tube where in the presence of external oxygen flash combustion occurs at a temperature of 1800 °C. The gaseous combustion products N_2, NO_x, H_2O, SO_2, O_2 and CO_2 are carried by the helium as carrier gas through a column filled with copper oxide and from there to a Cu-column where nitrogen oxides are reduced to elementary nitrogen, and O_2 to CuO. Water can be absorbed in another column. The remaining gasses are introduced into a TPD (Temperature Programmed Desoprtion) column where N_2 is going right through it and the other gases are bound to the column. With a programmed temperature raise in the column the gases are released separately. They flow along a thermal conductivity detector (TCD) which

produces an electrical signal proportional to the concentration of nitrogen, carbon, hydrogen and sulfur. Calibration is based on weight percent nitrogen in a single primary standard.

CNS methodology: Measuring the Total Amounts of Nitrogen, Carbon and Sulfur

For total Nitrogen, Carbon and Sulfur analysis, solid sample materials are converted to N_2, CO_2 and SO_2.

The general methodology is as follows:

1) A rotating auto-sample changer delivers one tin encapsulated sample at a time into the top of the quartz combustion tube.
2) This reaction tube, containing chromium oxide

d. Combustion catalyst and thin rods of copper, is held at 980°C. A pulse of pure oxygen (99.99% purity) is injected with each tin capsule. Thermal energy from the combustion of tin and sample material can generate a temperature of as much as 1700°C at the moment of flash combustion. All combustible materials in the sample are burned and the gas phase combustion products are swept out of the bottom of the combustion tube by a constant flow of non-reactive helium carrier gas.

3) All nitrogen bearing combustion products, including N2 and the various oxides of NOx, passes through the reaction column that has thin copper rods. The nitrogen oxides give up their oxygen and emerge from the column as N_2.
4) All of the carbon is converted to CO_2 and the sulfur is converted SO_2. Water vapor is removed from the sample as the gases pass through a magnesium perchlorate vapor trap. The clean sample gases pass through a Gas chromatography column that separates the N_2, CO_2, and SO_2. N_2 elutes first, at about 1 minute. CO_2 elutes second, at about 1.75 minutes. SO_2 elutes last, at about 7 minutes.
5) The sample gas pulses and the references stream of helium pass through a detector. The differences in the thermal conductivity between the two streams are displayed as viable peaks and are recorded as integrated areas
6) Linear regression is applied to combustion of known standard materials yields a regression line. This regression line is used to convert peak area of unknowns into total element values for each sample.

SAC 454

LECTURE 4.

SPECTROPHOTOMETRY: PRINCIPLE, TYPES AND INSTRUMENTATION

Objectives

1. To measure the absorbance of the sample at different wavelengths.
2. To find out the unknown concentration of the sample.
3. Verification of Beer-Lambert's Law.

What is spectrophotometry?

It is the quantitative measurement of how much a chemical substance absorbs light by passing a beam of light through the sample using a spectrophotometer. By measuring the intensity of light detected, this method can be used to determine the concentration of solute in the sample.

The spectrophotometer is the instrument used in biological, chemical, clinical and environmental research.

The basic principles involved are Beer's and Lambert's law.

a. Lambert's Law

When a monochromatic light (refers to visible light of narrow band of wavelength or light of a single wavelength e.g. Green/red laser light) passes through an absorbing medium, its intensity decreases exponentially as the **length** of the medium increases.

b. Beer's Law

The intensity of a ray of monochromatic light passing through an absorbing medium decreases exponentially as the **concentration** of the absorbing medium increases.

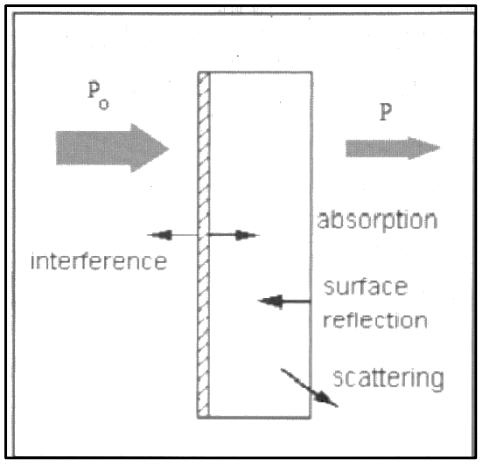
. Beer – Lambert's Law

The Beer –Lambert's law states that the concentration of a substance in solution is directly proportional to the absorbance 'A' of the solution.

Absorbance A = constant x concentration x cell length

Many compounds absorb ultraviolet (UV) or visible (Vis.) light.

The diagram below shows a beam of monochromatic radiation of radiant power P_0, directed at a sample solution. Absorption takes place and the beam of radiation leaving the sample has radiant power P.



The amount of radiation absorbed may be measured in a number of ways:

Transmittance, $T = P / P_0$

% Transmittance, $\%T = 100\ T$

Absorbance,

$A = \log_{10} P_0 / P$

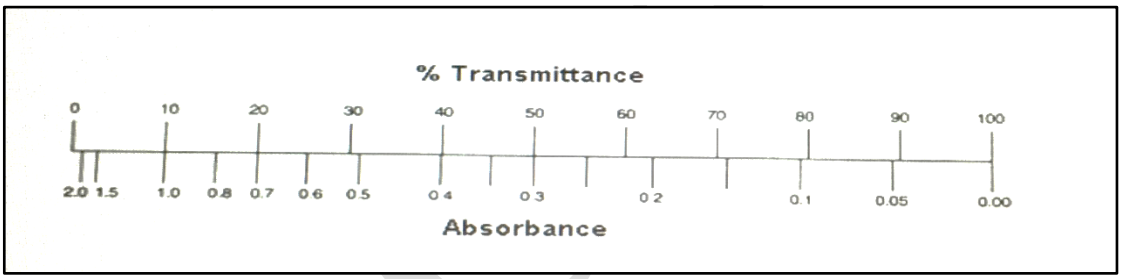
$A = \log_{10} 1 / T$

$A = \log_{10} 100 / \%T$

$A = 2 - \log_{10} \%T$

The last equation **$A = 2 - \log_{10} \%T$** , is worth remembering because it allows you to easily calculate absorbance from percentage transmittance data

The relationship between absorbance and transmittance is illustrated in the following diagram:



So, if all the light passes through a solution without any absorption, then absorbance is zero, and percent transmittance is 100%. If all the light is absorbed, then percent transmittance is zero, and absorption is infinite.

The ***Beer-Lambert law*** is:

$A=\varepsilon bc$

Where

A is absorbance (no units, since $A = \log_{10} P_0 / P$)

ε is the molar absorptivity with units of L mol^{-1} cm^{-1}

b is the path length of the sample - that is, the path length of the cuvette in which the sample is contained. We will express this measurement in centimetres.

c is the concentration of the compound in solution, expressed in mol L^{-1}

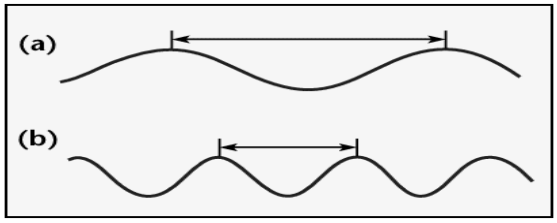
Electromagnetic spectrum

Light passes in the form of electromagnetic radiation. It is a form of energy transmitted as waves having electric and magnetic components. The visible light occupies only a small region in the spectrum of electromagnetic radiations.

Wave length is the distance between two maxima of an electromagnetic wave. It is expressed as metres, cm, mm, micron (1 in million) milli micron (mμ) nanometer (nm) and Angstrom (A^0) Units.

$1A^o = 10^{-8}$ cm = 10^{-10} m

$1nm = 10^{-6}$ mm = 10 A^o



(a) *longer wavelength, lower energy;*

The **major components** of electromagnetic spectrum are

a. Gamma ray region : 0.02-1A^o shortest waves emitted by atomic nuclei

b. X ray region : 1 – 10^0 A emitted due to movement of electron

c. Ultraviolet region : Vacuum ultraviolet 1-180 nm; ultraviolet - 180-400 nm

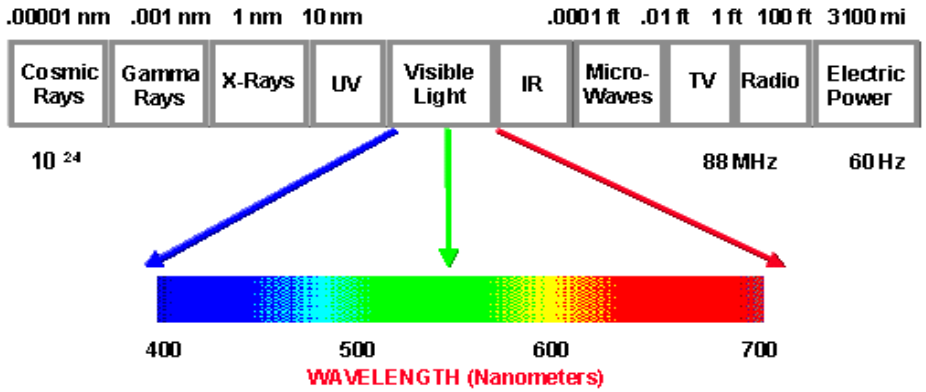
d. Visible region : 400-750 nm

e. Infra-red region : Near infrared (0.7-2.5 μ), Infrared (2.5-15.0μ), Far infrared (15-200 μ)

f. Microwave : 0.1 mm to 1 cm

g. Radiofrequency : 1cm to 10 m

Electromagnetic Spectrum



The energy of electromagnetic radiation is inversely related to the wavelength.

Visible range

Colour	Wave length (nm)	colour	Wave length (nm)
Violet	400-435	Yellow-green	560-580
Blue	435-480	Yellow	580-595
Green Blue	480-490	Orange	595-610
Blue green	490-500	Red	610-750
Green	500-560		

In the colorimetric and spectrophotometric methods, only solutions that absorb light can be used. The accuracy and sensitivity of the determinations will be greater when the light of greater intensity is absorbed.

Colorimeter

Colorimeter is a light-sensitive instrument that measures how much color is absorbed by an object or substance. It determines color based on the red, blue, and green components of light absorbed by the object or sample, much as the human eye does.

When light passes through a medium, part of the light is absorbed, and as a result, there is a decrease in how much of the light reflected by the medium. A colorimeter measures that change so users can analyze the concentration of a particular substance in that medium.

The device works on the ***basis of Beer-Lambert's law***, which states that "***the absorption of light transmitted through a medium is directly proportional to the concentration of the medium"***.

A colorimeter measures only red, green, and blue colors of light, while a spectrophotometer can measure the intensity of any wavelength of visible light. In general, spectrophotometers are more complicated and less rugged than most colorimeters; they should be handled with utmost care and require regular recalibration.

Components of a Colorimeter:

1) a source of radiant energy - An incandescent lamp with a tungsten filament
2) filter for isolation of a band of radiant energy
3) an optical system for producing a parallel beam of filtered light for passage through an absorption cell
4) sample container – cuvettes made of glass for visible region
5) a detector and
6) Read out meters

Mainly uses a filter to isolate the wave length. A photocell or a photo tube is used as a detector. It is used to measure absorption in the visible range.

What is spectrophotometer?

Spectrophotometer is used to measure the intensity of specific spectral line of the light.

It is a type of photometer that measures light intensity, is often grouped together with colorimeters, but it is technically a different device. Both rely on Beer-Lambert's law to calculate the concentration of a substance in a solution, but they do so in different ways.

- A spectrophotometer is an instrument that measures the amount of light absorbed by a sample.
- Spectrophotometer techniques are mostly used to measure the concentration of solutes in solution by measuring the amount of the light that is absorbed by the solution in a cuvette placed in the spectrophotometer.

- It is a device that can determine "how much color" is in a solution; this can be very useful in determining the concentration of the chemical that gives the solution its color.
- If a chemical is not itself colored, a dye may be added that bonds to the chemical of interest and forms color in the process. The spectrophotometer works by measuring how much light is transmitted (passed) through a solution.
- The solution is placed in a cuvette (a square glass or plastic vial) or a test tube, which is then placed into the machine.
- A light is shone through the cuvette / tube and onto a light detector, which changes the amount of light into an electronic signal. The light must pass through the two opposite smooth sides of the cuvette.
- Since a colored solution absorbs some of the light, the intensity of the light reaching the detector is lowered, or "absorbed".
- The amount of light that is absorbed by the solution depends on how many coloured molecules are in the path of the light, thus leading to Beer's Law.

Principle of Spectrophotometer

The spectrophotometer technique is to measure light intensity as a function of wavelength.

• It does this by:

- diffracting the light beam into a spectrum of wavelengths
- direct it to an object
- receiving the light reflected or returned from the object

SAC 454

- detecting the intensities with a charge-coupled device
- displaying the results as a graph on the detector and then the display device

1. In the spectrophotometer, a prism (or) grating is used to split the incident beam into different wavelengths.
2. By suitable mechanisms, waves of specific wavelengths can be manipulated to fall on the test solution. The range of the wavelengths of the incident light can be as low as 1 to 2nm.
3. The spectrophotometer is useful for measuring the absorption spectrum of a compound, that is, the absorption of light by a solution at each wavelength.

Components of a spectrophotometer:

Spectrophotometer consists of two instruments, viz., ***spectrometer*** for producing light of any selected color (wavelength), and a ***photometer***for measuring the intensity of light. The instruments are arranged so that liquid in a cuvette can be placed between spectrometer beam and photometer. The amount of light passing through the tube is measured by photometer which delivers a voltage signal to a display device, normally a galvanometer. The signal changes as the amount of light absorbed by the liquid changes.

The essential parts of a spectrophotometer are

1. Stable source of radiation or light source:

- **Sources of visible radiation:** An ***incandescent lamp with a tungsten filament*** is used as a source for ***visible region, near I.R and near UV***. It has a wavelength range of about 330 to 900 nm. It has long life about 1200h.
- **Source of ultra violet radiation:** The ***hydrogen and Deuterium lamps*** are most common sources of ***UV radiation in the range of 200 to 450 nm***. The tubes are filled with either H_2 or H_2 gas at low pressure. Deuterium lamps are generally more stable and has long life about 500h.This lamp generates continuous or discontinuous spectral.
- **Source of ultra violet and visible radiation:**Xenon flash lamps Xenon flash lamps have several advantages as the following:

  1) Their range between (190nm - 1000 nm)

  2) Emit both UV and visible wavelengths

  3) Long life

  4) Do not heat up the instrument

  5) Reduce warm up time
- **Source of infra-red radiation:*Nernst glower.*** It consists of a mixture of zirconium and yttrium oxides in the shape of a tube which is electrically heated to 1500-2000^{o}C.

2. Monochromator:

Dispersion devices/Dispersion devices causes a different wavelength of light to be dispersion at different angles. A monochromator is capable of resolving polychromatic radiation into its individual component wavelengths and isolate them into beams with very narrow bands. It transmits only narrow band of wavelength to pass through and is usually done by prisms.

- Visible region – glass prisms
- UV, near IR and visible – quartz prism
- Far infra-red – prism made of rock salt

Types of Dispersion devices used are ***Prism, Filters*** and ***Diffraction gratings***

Prism is used to isolate different wavelength. Prism may be made of glass or quartz. The glass prisms are suitable for radiation essentially in the visible range whereas the quartz prism can cover the ultraviolet spectrum also.

Filters separate different parts of the electromagnetic spectrum by absorbing or reflecting certain wavelengths and transmitting other wavelengths. Absorption filters are glass substrates containing absorbing species that absorb certain wavelength. A typical example is a cut on colour filter, which blocks short wavelength light, and transmits longer wavelength.

Diffraction grating is an optical component with a regular pattern, which splits (diffracts) light into several beams travelling in different directions. The diffraction grating disperses the light into a linear spectrum of its component wavelengths, which is then directed, in whole or in part along the light path of the instrument.

Monochromator consists of these part

I. Entrance slit
II. Collimating lens or mirror
III. Dispersion element
IV. Focusing lens or mirror
V. Exit slit

Dispersion devices: A special plate with hundreds of parallel grooved lines.

• The grooved lines act to separate the white light into the visible light spectrum.

The more lines the smaller the wavelength resolution.

Focusing devices:

Combinations of lenses, slits, and mirrors.

• relay and focus light through the instrument.

SAC 454

3. **Transparent sample holders / Sample container / Cuvettes:**

A cuvette is a kind of cell (usually a small square tube) sealed at one end designed to hold samples for spectroscopic experiments.

- made of plastic, glass or optical grade quartz

- should be as clear as possible, without impurities that might affect a spectroscopic reading.

- visible region - Glass cells cuvette
- UV region - quartz or special high silica glass
- IR region - rock salt cuvette

4. **Radiation detector**

– Any photosensitive device can be used as a detector of radiant energy. The photocell and phototube are the simplest photodetectors, producing current proportional to the intensity of the light striking them.

– Convert radiant energy (photons) into an electrical signal.

– The photocell and phototube are the simplest photo detectors, producing current proportional to the intensity of the light striking them

- Visible, UV region - Photoelectric detectors
- Infra-red region - Thermo electric detectors

5. **Amplifier**

6. **Read out system:** The data from a detector are displayed by a readout device, such as an analog meter, a light beam reflected on a scale, or a digital display, or LCD (liquid crystal display).

• The output can also be transmitted to a computer or printer

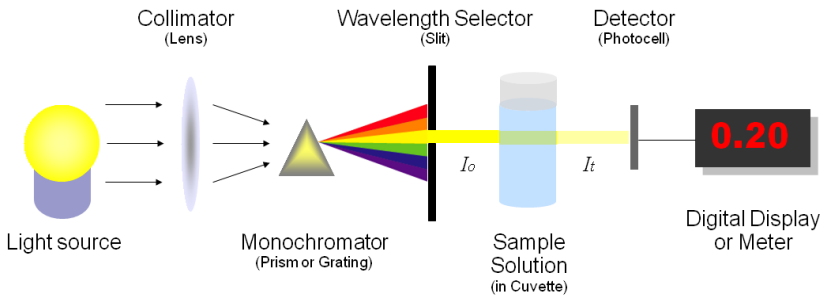


Figure 1: Basic structure of spectrophotometers (illustrated by Heesung Shim)

Referring back to ***Figure 1*** (and ***Figure 2***), the amount of photons that goes through the cuvette and into the detector is dependent on the length of the cuvette and the concentration of the sample. Once you know the intensity of light after it passes through the cuvette, you can relate it to transmittance

(T). Transmittance is the fraction of light that passes through the sample. This can be calculated using the equation:

$$\text{Transmittance (T)} = I_t/I_o$$

where I_t is the light intensity after the beam of light passes through the cuvette and I_o is the light intensity before the beam of light passes through the cuvette. Transmittance is related to absorption by the expression:

$$\text{Absorbance (A)} = -\log (T) = -\log (I_t/I_o)$$

where absorbance stands for the amount of photons that is absorbed. With the amount of absorbance known from the above equation, you can determine the unknown concentration of the sample by using Beer-Lambert Law. **Figure 2** illustrates transmittance of light through a sample. The length ***l*** is used for Beer-Lambert Law described below.

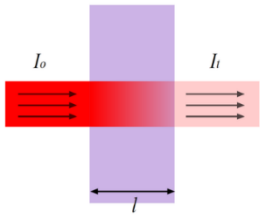


Figure 2: Transmittance (illustrated by Heesung Shim)

Types of Spectrophotometers

A photoelectric colorimeter uses a tungsten filament lamp as a source which gives radiations in the visible region (400-700 nm). In a spectrophotometer such energy sources can be used to give out radiations in visible as well as in infrared and ultraviolet regions. If an infrared source is used, the instrument is known as an **I.R. spectrophotometer**. An instrument which is having both visible and ultraviolet radiation sources, is called as **UV - Vis spectrophotometer**

1. Infrared Spectrophotometer

In this instrument, the source of radiant energy is Nernst glower. It consists of a mixture of zirconium and yttrium oxides in the shape of a tube which is electrically heated to 1500-2000^{o}C. Because infrared rays are not transmitted by glass, a prism made of salt (NaCl) is used as a monochromator. The radiations from the Nernst glower are polychromatic and when passed through the salt prism, the wavelengths get separated. Thermal detectors are used for measuring the intensity of transmitted infrared radiations. An I.R. spectrophotmeter can give the absorption spectrum of a substance. By analysing this spectrum, information regarding the structure of substance can be obtained.

2. U.V -Vis Spectrophotometer

This type of spectrophotometer covers the range of **220-1000nm**, which includes ultraviolet, visible and near infrared regions. The intensity of the beam transmitted by the sample solution is

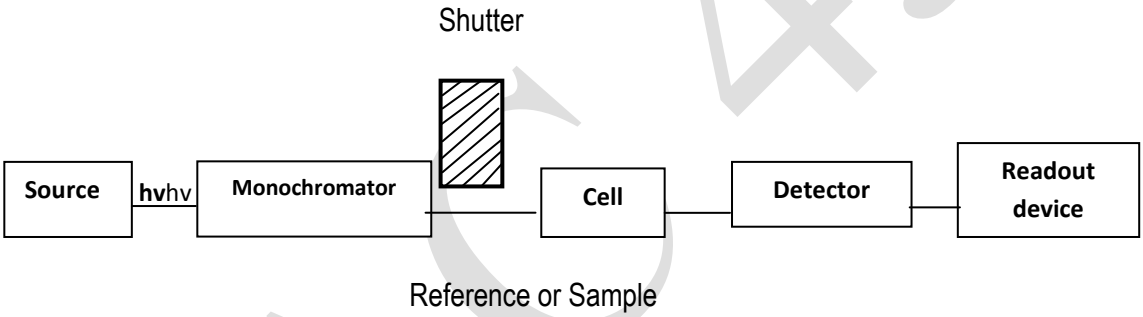
measured by a photocell. A glass prism cannot be used because glass does not transmit ultraviolet light. A silica prism is suitable for both, visible and ultraviolet radiations.

Types of Ultra violet and visible spectrophotometers

i) Single beam spectrophotometer

A single beam instrument consists of a source of light, a monochromator, two matched cells through which the radiation is passed alternately at each wavelength, a photomultiplier detector and a readout device.

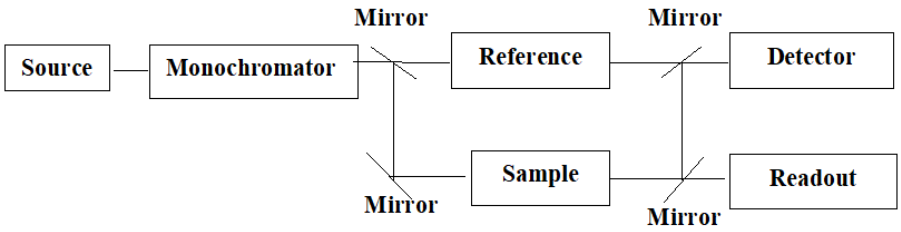
Initially 0 % transmittance archived by placing the shutter in between the monochromator and cell and the reading in the readout device is adjusted mechanically to zero transmittance value. Next, the shutter is removed and the light is allowed to pass through the reference cell and the reading in the readout device is adjusted to 100 % transmittance. Finally, light is allowed to pass through the sample and the reading shown in the readout devices is taken as a transmittance of the sample. The whole process is repeated for each wavelength used to get the absorbance spectrum of the sample.



Schematic Diagram of a Single-beam Instrument

ii) Double beam spectrophotometer

In a double beam instrument with beams separated in time, light from the source passes alternatively through the reference and sample cells. This is done by a ***rotating mirror /chopper*** which get into and out of the radiation path. When the chopper allows the entire beam to pass through sample the detector measures the intensity of the light as I. When the chopper directs the light to pass through the reference cell the detector measures the intensity of light as Io. The two intensities are compared by the instrument to register the absorbance of the sample.



Schematic Diagram of a Double-beam Spectrophotometer (beams separated in time)

In transmission measurements, spectrophotometer quantitatively compares the amount of light passing through the reference and test sample. For reflectance, it compares the amount of light reflecting from the test and reference sample solutions.

Many spectrophotometers must be calibrated before they start to analyze the sample and *the procedure for calibrating spectrophotometer is known as* ***"zeroing."*** Calibration is done using the reference substance, and the absorbencies of all other substances are measured relative to reference substance. % transmissivity (the amount of light transmitted through the substance relative to the initial substance) is displayed on the spectrophotometer.

Instrumentation:

Operation of the Spectrophotometer

1. Before beginning the measurement, turn on the spectrophotometer, by rotating the Power Switch/Zero Control knob clockwise (see Fig. 1). This should be done 10 min before the measurements are to be made to give the machine enough time to **warm up**. This will allow the lamps to warm up for an appropriate period of time to stabilize them.

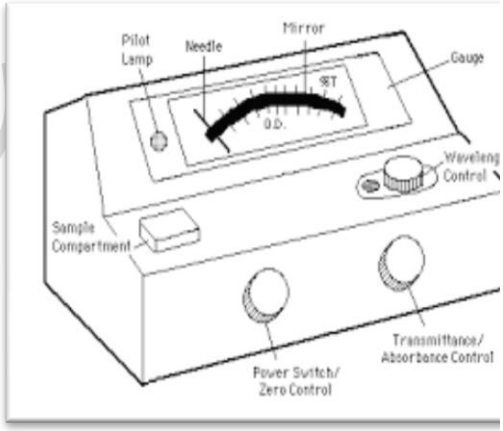


Fig. 1.Spectrophotometer

2. Select the proper wavelength. Turn the Wavelength Control knob, which is on top of the instrument, so that it registers the wavelength at which you want to measure your sample.
3. Adjust meter needle to "0" (left margin) by turning the Zero Control knob on the left
4. Prepare a blank by filling a clean cuvette with the sample solvent, and then wipe the outside with lint-free paper to remove any fingerprints.

5. Insert the cuvette containing 5 ml of control blank into the sample compartment. Ensure that the cuvette is aligned properly with the grooved sides out of the beam-path, and insert it into the spectrophotometer. Secure the lid to prevent ambient light from entering the system.
6. Adjust the meter needle to 100% transmittance by turning the Transmittance/ Absorbance Control knob (on the right). This knob regulates the amount of light passing through the exit slit. Now that the meter is adjusted for zero and 100 % transmittance, turbidity measurements can be made.
7. Measure the absorbance of the blank at one wavelength, or over a wavelength range. Record or save the absorbance, as it must be subtracted from the absorbance of the sample.
8. Remove the blank and rinse the cuvette twice with sample. Then, fill the cuvette about ¾ full with the sample to be read. Wipe the outside of the cuvette again, to ensure that it is clean and free of fingerprints.
9. Place the cuvette in the spectrophotometer in the correct orientation, and secure the lid.
10. Collect an absorbance measurement or spectrum [i.e. note the O.D. (bottom scale) or percent transmittance (top scale)]at the same wavelength or wavelength range as the blank. Subtract the blank spectrum or measurement, if the instrument does not automatically do so.
11. From the collected absorbance spectrum, determine the absorbance maximum, or λ max.
12. To quantify the amount of analyte in the sample, construct the calibration/standard curve using a range of known analyte concentrations.
13. Continue to read any other samples you may have. If you have a large number of samples to measure, recheck and adjust the transmittance using your blank after every ten readings.
14. When you have finished your measurements, turn off the spectrophotometer, remove your blank or sample and clean the instrument.

APPLICATIONS:

Some of the major applications of spectrophotometers include the following:

- Detection of concentration of substances
- Detection of impurities
- Structure elucidation of organic compounds
- Monitoring dissolved oxygen content in freshwater and marine ecosystems

- Characterization of proteins
- Detection of functional groups
- Respiratory gas analysis in hospitals
- Molecular weight determination of compounds
- The visible and UV spectrophotometer may be used to identify classes of compounds in both the pure state and in biological preparations.

References

1. https://www.biochemden.com/spectrophotometer-instrumentation-principle/
2. https://www.azom.com/article.aspx?ArticleID=10245
3. https://web.stanford.edu/class/chem184/lectures08/Zare_Spectroscopy.pdf
4. https://byjus.com/chemistry/spectrophotometer-principle/
5. https://www.slideshare.net/rey216/spect
6. https://www.slideshare.net/AMASM/spectrophotometer
7. https://di.uq.edu.au/community-and-alumni/sparq-ed/sparq-ed-services/spectrophotometry
8. https://www.jove.com/v/10204/ultraviolet-visible-uv-vis-spectroscopy

SAC 454

LECTURE 5

SPECTROPHOTOMETRY: SAMPLE HANDLING AND MEASUREMENT, METHOD DEVELOPMENT AND VALIDATION AND CHECKING FOR ACCURACY

Spectrophotometer is a method used to estimate the level of an analyte in solution. It relies on the principle that materials absorb light of a certain wavelength as it passes through the solution. ***Generally, the spectrophotometer is used to determine the concentration of P, S and barium in soil, water and fertilizer samples***.

Sample handling and measurement by UV-Spectrophotometry involves:

- Preparation of stock solution
- Preparation of sample solution in 5-6 replicates by weighing the sample individually
- The stability of analyte in suitable diluent solution was studied by UV method
- Preparation of sample solutions in triplicate and stored at 4 & 25⁰ C for different durations (e.g. 30, 60, 90 & 120 min.)

- Beer's Law states that the amount of light of a particular wavelength absorbed by a substance across a constant distance (light path) is proportional to the concentration of that substance.

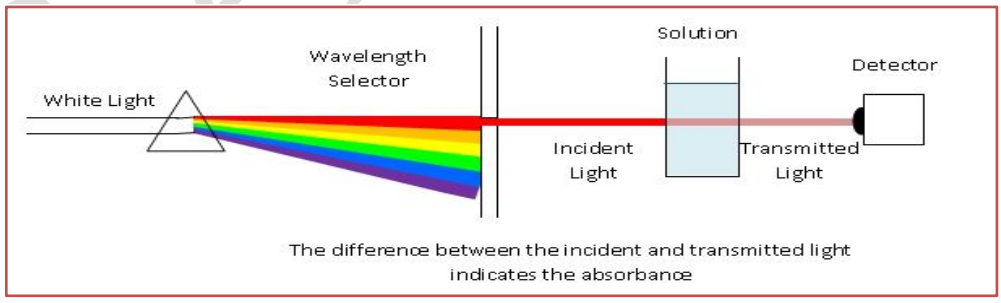
  ie. $A_n = l \times c$

  where, A_n is the absorbance at n nm

  l is the light path (cm)

  c is the concentration

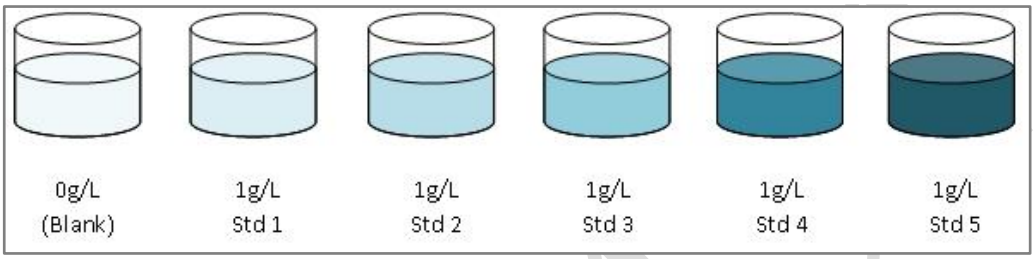
- A spectrophotometer is a device which measures the absorbance of a solution as light of a specified wavelength is passed through it.



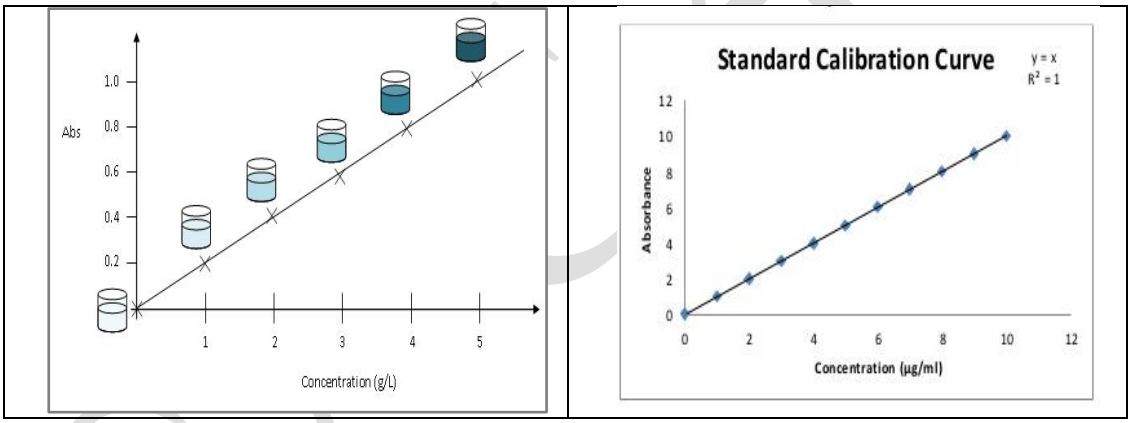
- If we measure the absorbance of a solution containing a known concentration of an analyte, we can use this value to estimate the concentration of the analyte in an unknown solution by comparing the two absorbance values
- The range over which absorbance is proportional to concentration varies according to the analyte and the wavelength of light used. To ensure that there is a direct relationship between

absorbance and concentration, we must prepare a standard curve. Despite its name, the part of the standard curve that gives a proportional relationship is a straight line.

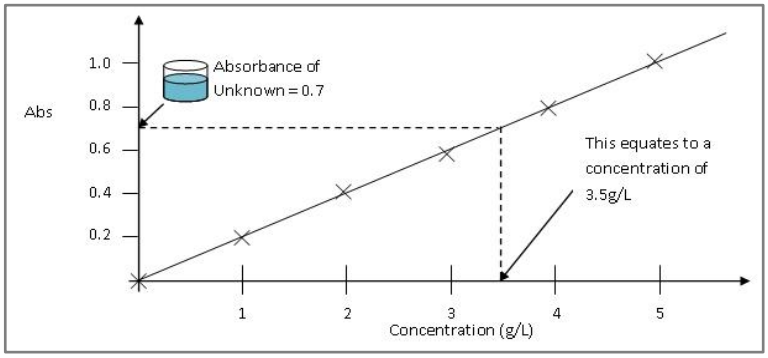
- To prepare a *standard calibration curve*, we prepare a series of dilutions of a standard solution of our analyte of known concentration. The first tube always contains none of the analyte (eg. a concentration of 0g/L) and we call this a ***blank***. We use this to calibrate the spectrophotometer to take into account the natural absorbance of the diluents. Each of the following tubes contain increasing concentrations of the analyte. The absorbances of each of the standards are read using the spectrophotometer.



- To create the standard curve, we plot a line graph of Absorbance (Y axis) vs Concentration (X Axis) for each of the standards. A line of best fit is then drawn through the points.



- To estimate the concentration of an unknown solution of the analyte, we read the absorbance, and then use the standard curve.



Most analytical methods require calibration, a process that relates the measured analytical signal to the concentration of analyte, the substance to be analyzed.

The three most common ***Calibration techniques include***

- Calibration curve method,
- Standard additions Method
- Internal standard method

Calibration curve method - Advantages

- Most convenient when a large number of samples are to be analyzed
- Most common technique
- Facilitates calculation of figures of merit

In analytical chemistry, the ***calibration curve method*** is a general method for determining the concentration of a substance in an unknown sample by comparing the unknown to a set of standard samples of known concentration. In spectrophotometric analysis a series of standard solutions of known concentrations are prepared and absorbance is measured using spectrophotometer instrument to determine the unknown concentration of sample by Beer's Law.

A calibration curve is a graph where concentration is plotted against absorbance then a straight line (Beer's Law) is fit to the data that we obtained and the resulting equation is used to convert absorbance of the unknown sample into concentration

1. Calibration curve procedure

1. Prepare a series of standard solutions (analyte solutions with known concentrations).
2. Plot [analyte] vs. Analytical Signal.
3. Use signal for unknown to find [analyte].

2. Standard additions Method

1. Most convenient when a small number of samples are to be analyzed.
2. Useful when the analyte is present in a complicated matrix and no ideal blank is available.

3. Internal Standard Method

1. Most convenient when variations in analytical sample size, position, or matrix limit the precision of a technique.
2. May correct for certain types of noise.

Method development:

It is defined as a process of formulating the materials, conditions and protocol for measuring the analyte.

SAC 454

Steps involved in method development using UV spectrophotometer

I. **Selection of solvent system**
   - The stability of analyte in suitable diluent solution was studied by UV method
   - Sample solutions were prepared in triplicate and stored at 4 & 25^0 C for different durations (e.g. 30, 60, 90 & 120 min.)

II. **Selection of analytical wavelength**
   - Proper wavelength selection of the methods depends upon the nature of the sample & its solubility.
   - To develop a rugged & suitable spectrophotometric method (UV), the analytical condition where selected after testing different parameters such as diluents, buffer & buffer concentration.
   - Standard solutions of known concentrations were prepared and absorbance was measured in UV spectrometer between 200 - 400nm on spectrum mode, using diluents as a blank.
   - Then standard calibration curve was prepared

III. **Study of beers lambert's law**

   The assay of single component sample, which contains other absorbing substances, is then calculated from the measured absorbance by using calibration graph procedures.

IV. **To perform analysis of standard laboratory mixture & analyte sample by proposed method.**

V. **To validate the developed methods by using Different validated parameters.**

   Once the method is developed, it should be ***validated*** for its application for ***its accuracy, linearity, robustness, sensitivity and selectivity***.

Validation:

It is defined as establishing documented evidences which provides a high degree assurance that a specific process will consistently produce a product meeting its pre-determined specification & quality characteristics.

Once the method is developed, it should be validated for its application for the validation parameters viz., ***accuracy, precision, limit of detection, limit of quantitation, specificity, linearity, range, robustness, ruggedness sensitivity and selectivity***.

Validation parameters:

Precision and accuracy is determined with standard quality control sample prepared in triplicate at different concentration levels**.**

Accuracy:

The closeness of agreement between the value which is accepted as a conventional true value or an expected reference value, and the value found.

- Accuracy is the percentage of analyte recovered by assay from a known added amount.
- Data from 9 determinations over 3 concentration levels covering the specified range.

Precision:

It expresses the closeness of agreement between a series of measurements obtained from the multiple sampling of the homogenous sampling under the prescribed conditions.

- Precision of the assay was determined by repeatability and intermediate precision, which is studied by comparing the assays on 3 different days and results are documented

Precision may be considered at 3 levels: *repeatability, intermediate precision, reproducibility*.

1. **Repeatability**: it expresses the precision under the same operating conditions over a short interval of time.
2. **Intermediate precision:** It expresses with in laboratory variations: different days, different analyst, different equipment
3. **Reproducibility:** It expresses the precision between laboratories.

Limit of detection (LOD):

Lowest concentration of analyte present in a sample that can be detected and which an analytical process can reliably differentiate from background levels.

Limit of quantitation (LOQ):

It is the lowest concentration of analyte in a sample which can be quantitatively determined with an acceptable accuracy, precision and variability.

Specificity: It is the ability to assess the analyte in the presence of components which may be expected to be present. It includes impurities, degradants, matrix etc.

This definition has following implications:

Identification: To ensure the identification of analyte

Purity test: To ensure an accurate content of impurities of an analyte i.e. heavy metals, residual solvent content.

Assay: To provide an exact result this allows an accurate statement on content or potency of analyte in the sample.

Linearity: Its ability (with in a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

- It is obtained by generating a5-point calibration curve.

Range: It is the interval between upper and lower concentration of analyte in the sample.

SAC 454

Robustness: It is a measure of its capacity to remain unaffected by small variations in method parameters and provides an indication of its reliability during normal usage.

Ruggedness: Reproducibility under normal but variable laboratory conditions.

References

1. https://di.uq.edu.au/community-and-alumni/sparq-ed/sparq-ed-services/spectrophotometry
2. https://www.jove.com/v/10204/ultraviolet-visible-uv-vis-spectroscopy
3. Analytical &bio analytical techniques-SiladityaBehera, 2012.
4. Asian Journal of Pharmaceutical and Clinical Research Vol-6, Suppl-3, 2013.
5. Principles of instrumental analysis- Skoog DA, Holler FJ, 6th Edition.
6. International Journal of Pharmaceutical Science & Research Vol.1(2),2010,122-126.
7. The Merck Index, Merck Research Laboratories division of Merck and company, 13th ed., NJ, USA, 2001, 1627

SAC 454

LECTURE 6

EMISSION SPECTROSCOPY (FLAME PHOTOMETER): INSTRUMENTATION, INTERFERENCES, TROUBLE SHOOTING AND MAINTENANCE

Absorption and Emission

Absorption techniques measure the absorbance of light due to the electrons going to a higher energy level. **Emission techniques** measure the intensity of light that is emitted as electrons return to the lower energy levels.

Emission spectroscopy

When the sample is excited electrically to burn in an arc or thermally in a flame it produces / emits spectrum characteristic of its component metals (emission spectrum) which when passed through a dispersing system of a spectrograph, the intensity of individual lines spectrum can be measured. Thus all the elements present are measured quantitatively.

FLAME PHOTOMETERY / FLAME EMISSION SPECTROSCOPY

It is the study of the photon (light) energy emitted when a metal is introduced into the flame. Radiation in the visible and ultra violet regions occur when the atoms or molecules are excited by the absorption of energy.

Principle

When an alkali metal is excited in a non luminous flame, electrons move to the excited state in the flame and when they return to the ground state, they emit radiations of characteristic wavelength in the form of photons. **The intensity of emission is proportional to the concentration of the element in the solution.** Each element emits radiation at a wavelength specific for that element.

Element Emission Wavelength (nm) & Flame Colour

Element	Emission Wavelength (nm)	Flame Colour
Sodium (Na)	589	Yellow
Potassium (K)	766	Lilac
Barium (Ba)	554	Lime Green
Calcium (Ca)	622	Brick Red
Lithium (Li)	670	Red

Components of a Flame photometer

Flame photometer consists of the following parts

Atomizer

This is a device to introduce the liquid sample in to the flame for conversion into vapour form. The passage of oxygen from the tip causes the solution to be drawn up to inner capillary tube where it is

dispersed into fine droplets. Appropriately 1-2 ml of the sample is consumed in 1 min. This device must introduce the sample into flame at a stable and reproducible rate.

Burner

The main requirement of burner is when supplied with fuel and air at constant pressure, it should produce a steady flame. The flame produced

a) Transforms the sample to be analysed from liquid state into gaseous state.
b) Decomposes molecular compounds of element into simpler molecules or atoms
c) Excites the atoms to light emission.

Pressure regulators and flow meters for fuel gases

Pressure or flow rate of the gas is controlled by pressure regulator. Suitable gauges indicate the pressures and flow rates actually prevailing when the instrument is in operation. A 10 lb gauge for the fuel and a 25 1b gauge for the oxygen or air supply are generally satisfactory. A change in quantity of fuel or air flowing into the burner varies the flame characteristics and there by reproducibility of results is affected.

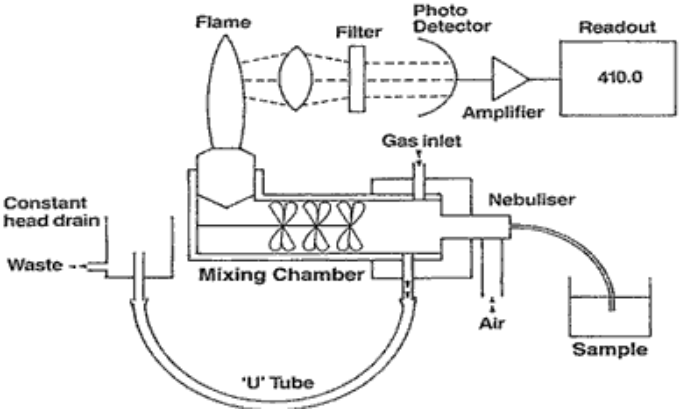
Optical system

The optical system collects the light from steadiest part of the flame, converts it into monochromatic, and then focuses it to the surface of photosensitive detector.

Photosensitive detectors

Photocell or photomultiplier tube detection system converts the photons / light energy into electric pulses and measured. Photo cell detector is used when a wide band of radiant energy strike the detector. When the band width reaching the detector is less, phototubes can be used to amplify and to detect the radiation.

Flame Photometer



Schematic Sketch of a Flame Photometer

OPERATION

The liquid containing metallic salt solution is introduced into a flame, the following sequence of events occur

- Water or other solvent is vaporized, leaving a minute particle of dry salt.
- At high flame temperature, the dry salt is vaporized and the gaseous molecules are dissociated to give neutral atoms.
- The vapors of neutral atoms are excited by thermal energy of flame
- The excited atoms are unstable and return back to their original position by spontaneous emission of light
- Collection and detection of emitted light energy and conversion into electric pulses.

Factors influencing the intensity of radiation emission

- Viscosity – It decreases the intensity of light emission due to the reduction in atomization
- Presence of acids – It decreases the light intensity by disturbing the dissociation equilibrium
- Presence of other metals – Alter the intensity of radiation
- Flame Temperature – Excitation of atoms depend on temperature

Interferences / Disadvantages of Flame Photometer

- **Spectral interferences -** Arise from the close proximity of other emission lines or bands which can be minimized by increasing the resolution of instrumentation. Eg. Changing from filter photometer to grating spectrophotometer
- **Chemical interference by other elements -** The presence of species in the flame other than those of the analyte may alter the emitted intensities of analyte through chemical interactions. Eg. Sulfate, nitrate and phosphate. Addition of chelating agents is the better way of eliminating such effects as the chelating agents protects the metal from the interfering ion.
- **Self-absorption -** It is a phenomenon where by emitted radiation is reabsorbed as it passes outwards form the central region of the flame.
- **Fluctuation in flame temperature -** Intensity of emission is very sensitive to changes in flame temperature

Fuel	Oxidizing agent	Temperature °C
Hydrogen	Air	2100
Propane	Oxygen	2775
Natural gas	Air	1700
Natural gas	Oxygen	2700
Acetylene	Oxygen	3200

Merits

SAC 454

Flame photometers are generally employed for estimating alkali and alkaline earth metals (sodium, potassium, calcium and lithium, rubidium, caesium, barium) for which the chemical methods are time consuming.

MAINTENANCE

Generally maintenance requirements are minimal. To maintain good performance and prolong the life of the instrument it is important that the procedures defined in the manual are carried out regularly. The performance of the instrument depends upon an adequate supply of compressed air. Recommended compressor maintenance procedures should be carried out to ensure that compressor performance does not deteriorate.

Weekly maintenance

For ensuring efficient nebuliser operation, switch on the air supply to the flame photometer but do not light the flame and feed deionised water and present to the nebuliser inlet tube for one minute.The consumption rate should be between 2 and 6ml/min. If correct, no further action is required. If the consumption rate is too low, this is likely to be caused by a blockage in the fine capillary tubes and can usually be cleared by passing the cleaning wire through the nebuliser. If, after taking this action and re-checking the consumption rate it is still too low, then the inlet tube should be discarded and the test repeated using fresh tubing. If the nebuliser operation is still unsatisfactory, remove the nebuliser from the mixing chamber, disconnect from the air line, remove the inlet tubing and soak the nebuliser in hot deionised water. Refit the nebuliser and repeat the test. If operation is still unsatisfactory, a new nebuliser should be fitted.

TROUBLESHOOTING

Check the instrument stability is within the limits of the instrument specifications by checking against the reproducibility, linearity, specificity and stability parameters.

Possible cause	Solution
Condensation in the air supply	Drain the tubing. If the problem persists a moisturefree air supply should be arranged by using a water separator.
Nebuliser blocked.	Perform maintenance procedure
Mixing chamber/burner contaminated.	Ensure the instrument is always flushed with deionised water before shutdown.
Calibration standard at incorrect concentration or incorrectly diluted.	Make new standards to check the calibration procedure
Calibration curves incorrect.	Check the validity of the calibration curves using fresh standards.

Fuel regulator pressures incorrectly set.	Replace cylinder or reset regulator.
Burner encrusted with salt.	Rinse the burner in deionised water and remove any deposits or encrustations by brushing with a small stiff brush. If deposits are persistent, heating or boiling the deionised water will help.

REFERENCES

- https://www.bwbtech.com/post/2019/01/03/how-to-maintain-a-flame-photometer
- https://www.studyread.com/flame-photometry/
- http://www.jenway.com/adminimages/T05_002_Troubleshooting_Flame_photometer_ignition.pdf

SAC 454

LECTURE 7

ABSORPTION SPECTROSCOPY (ATOMIC ABSORPTION SPECTROPHOTOMETER): PRINCIPLE, INSTRUMENTATION, FEATURES AND OPERATION OF COMPONENTS

ATOMIC ABSORPTION SPECTROPHOTOMETER (AAS)

It is a method of determination of an element in a sample by measuring the absorption of radiation by the atomic vapour produced from the sample at a wavelength that is specific and characteristic of the element under consideration. The basis is the absorption of energy by neutral atoms in ground state when they are in gaseous form. Absorption is proportional to the density of atoms in the flame

Atomic Absorption Spectrophotometer is commonly used to estimate micronutrients like iron, manganese, zinc, copper, molybdenum etc.,

Principle

When light form a suitable source is directed through the atomic cloud of the sample, the detector measures the quantity of light absorbed by measuring the intensity of radiation before and after it passed through the sample. The atoms of sample, which is converted into gaseous form absorb energy in proportion to the density of atoms. The change in intensity of light gives the amount of energy absorbed by the atoms.

Components of AAS

1. Radiation source (Hollow cathode lamp)
2. Sample vaporizing units (Nebulizer, Atomizer, burner)
3. Monochromator (select wavelength of target element)
4. Detector
5. Deuterium continuum radiation source
6. Recorder

1. Radiation source

The radiation source should produce spectral lines having half width than that of the corresponding absorption lines. The most suitable and widely used source is **hollow-cathode lamp** (HCL) and to some extent micro-wave excited **electrodeless discharge tube** can also be used.

1a. Hollow Cathode Lamp

HCL consists of a sealed glass envelope with a quartz end window, and a hollowed-out cylindrical cathode of 2 mm internal dia together with a tungsten wire anode. The cathode is fabricated from the element to be determined. By reducing the pressure inside the envelope and passing a current

SAC 454

of 5-50 mA at an applied potential of 300 V, a low pressure glow-discharge characteristic of the cathode material is produced. The gas filled in the tube will bombard the cathode and thereby vaporize the atoms from the surface and this release of atoms form the cathode is known as **Sputtering.** Hollow cathode lamps are currently available for over sixty elements. In practice, neon or argon is preferred for hollow cathode lamp.

Ib. Electrodeless discharge tubes

Radiation is derived from a sealed quartz tube containing few milligrams of an element or a volatile compound and neon or argon at low pressure. The discharge is produced by a micro-wave source.

2. Sample vaporizing units

The sample solution is drawn first into a nebulizer by the flow of support gas where it forms a mist or aerosol. The sample droplets are introduced to the burner for combustion. The burner consists of a metal block containing a row of circular holes about 10cm long. A moderately hot flame (2400 K) from air-acetylene keeps practically 100% of atoms in ground state. The flame from normal LPG gas exceeds 3000 K, and so acetylene is used as fuel in AAS. The mixture of air- propane is still cooler and mixture of nitrous oxide and acytelene is hotter and is used for samples containing refractory materials.

The samples can also be vaporized by a graphite furnace or carbon rod instead of flame. Here, the temperature is raised to 2500 K by the passage of heavy current for a period of 1-25 minutes.

3. Monochromator

The purpose of the monochromator is to isolate a particular emission line from a number of characteristic lines emitted by the hollow cathode lamp.

4. Deuterium continuum radiation source

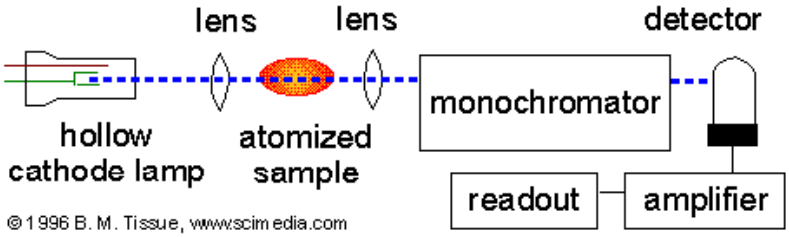
A deuterium continuum radiation source is used for background correction. Background error arises from the following. 1. Atoms in the flame get excited and emit radiation. 2. Electrons from the hollow cathode lamp emit some radiation. Continuous spectrum from deuterium lamp is used to compute correction.

5. Detectors

Photomultiplier tubes detect the difference in intensity of light before and after passing through the flame and expresses as absorbance. The amount of absorbance by the sample can be referred to the calibration curve and the concentration of the element present in the solution can be derived

Disadvantages

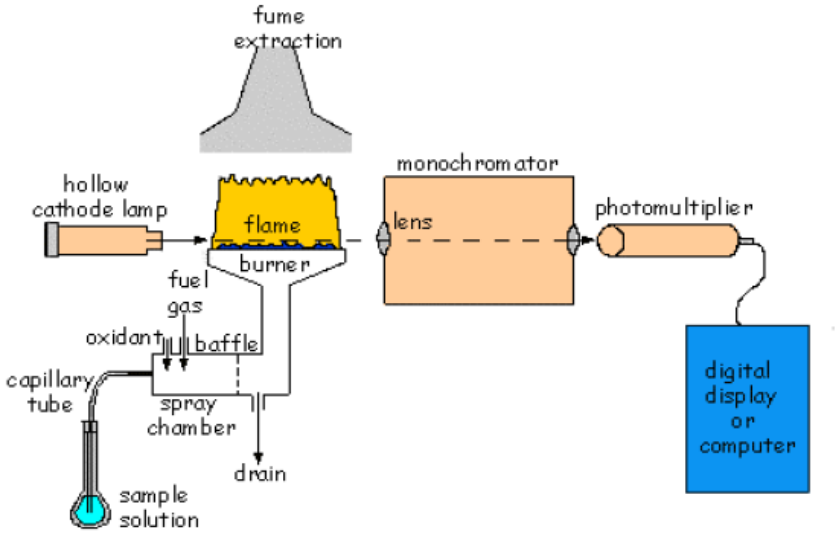
Individual source lamps are required for each element.



Schematic sketch of Atomic Absorption Spectrophotometer (1)

Advantages of Emission Spectroscopy

- Appreciable method for trace element analysis
- All metals and metalloids can be measured
- No chemical separation is needed. Samples can be analysed as such
- A very small sample is sufficient (1-10 mg)
- Time required is very small



Schematic sketch of Atomic Absorption Spectrophotometer (2)

Atomic Absorption Spectrophotometer

LECTURE 8

ABSORPTION SPECTROSCOPY (ATOMIC ABSORPTION SPECTROPHOTOMETER): SAMPLE HANDLING AND MEASUREMENT, ERRORS DUE TO MOLECULAR AND IONIC SPECIES, MATRIX EFFECT AND OTHER INTERFERENCES, TROUBLE SHOOTING AND MAINTENANCE

Elemental analysis of the majority of organic and inorganic matrices requires the partial or total dissolution of the sample prior to instrumental analysis. Analysis by spectroscopic methods practically always necessitates a simple or more complex preparation of the sample. These steps are generally the most critical part of analysis because they are responsible for the most important errors.

SAMPLE PREPARATION

Only a few direct methods allow the introduction of the sample without any preparation. In these cases the lack of reliable calibration is the major problem. On the other hand, sample preparation allows the separation and/or pre-concentration of analytes and makes possible the use of several determination methods. Sample preparations involve digestion, extraction and preparation of the analytes before the analysis.

Apparatus like hot plate, microwave digestion, acid digestion, high-pressure digestion can be used for sample preparation using acids like HNO_3, HCl, H_2SO_4, HF, H_2O_2. A typical sample preparation procedure for solid and viscous liquid samples involves digestion with a concentrated acid; for example, HNO_3, HCl, or H_2SO_4. After dilution of the digested solutions, samples can be directly injected into flame AAS as well as graphite furnace AAS. Other sample preparation methods, including microwave and high-pressure digestion, are also used to break up samples.

MEASURMENT

Atomic absorption spectrometry (AAS) is a globally recognized analytical technique used for analyzingover 60 elements including sodium, potassium, calcium, magnesium, zinc, and iron. It is widely accepted in many industries, which continue to utilize the unique and specific benefits of this technology.

During the analysis, liquid samples are aspirated and introduced into the flame via a spray chamber, which breaks the aspirated liquid into fine droplets. The flame is typically created using air/acetylene or nitrous oxide/acetylene gases, and this results in desolvation, vaporization, and atomization of the sample. Hollow cathode lamps emit light that is specific to the element, and this light is directed through the flame to allow for measurement during atomization. High-performance optics and precise monochromator operation ensure that the light path is always perfectly aligned for analysis.

ERRORS

The common errors in AAS are

- Changes in lamp intensity, contamination introduced during sample preparation and scattering of light by particles of extraneous material in the flame.
- Error is due to statistical variations in photon arrival at the photocell.
- Too, must electronic (Johnston) noise and flame (emission) noise, both of which produce an uncertainty in estimating the value of the signal, an uncertainty which is independent of per cent transmission.
- Changes in the apparent sensitivity of the analyte caused by fluctuations in flame temperature or gas mixture, and monochromator drift.

INTERFERENCES

Spectral and Chemical Interferences

- Absorption or emission of an interfering species overlaps or lies so close to the analyte absorption or emission that resolution is not possible. Rare with HCLs.
- Presence of combustion products that exhibit broadband absorption or particulates that scatter radiation. Both diminish power of transmitted beam and lead to positive errors. If caused by fuel/oxidant mixture, then correction is possible by running a blank and performing background subtraction. More troublesome problem when absorption or scattering results from the sample matrix.
- Interference by anions that form low volatility complexes with the analyte, and thus reduce the atoms formed. Lead to negative errors. Can be corrected by – Releasing agents (cations added to preferentially react) – Protecting agents (e.g., EDTA added to protect analyte cation)
- Dissociation unequilibriaand Ionization unequilibria- In both cases, analyte atoms are not all in the proper form to absorb or emit at desired wavelength. Lead to negative errors.

MAINTENANCE

- Check that there is enough gas in the cylinders at the start of the run, especially important for the acetylene to make sure that there is no carryover of acetone into the gas box of the instrument.
- Check that the exhaust is working correctly.
- Check the nebulizer uptake rate and inspect the burner to make sure it's clean and OK for analysis.
- At the end of the run, follow the recommended shut down procedure to wash out the sample introduction system, and then empty the waste containe

- It's also good practice to wipe down the exterior surfaces of your atomic absorption system especially in the sample compartment to remove any acid residue that may have built up on those surfaces.
- On a weekly basis or perhaps as necessary, clean both the burner and the flame atomization system. While doing that, inspect the components, particularly the impact bead and the condition of all the O-rings, to make sure they're in good condition. If the O-rings are knicked, damaged or obviously stretched, replace them immediately otherwise they won't do their job and won't give you a good seal.
- Periodically check the optical windows on the instrument and clean as necessary.

TROUBLE SHOOTING

Problem	**Causes**	**Rectification**
Low Absorbance	Nebulizer may be partially blocked Particulates in solution which contain the analyte A fault in the hollow cathode lamp	To reduce risk of blocking, nebulize the sample alternately with the blank solution at intervals of approximately 15 seconds. Frequently shake solutions containing particulate matter to ensure complete dispersion throughout the liquid. Lamp change.
Excessive Noise or Unstable Signals	self absorption broadening", in which the atoms in the hollow cathode lamp absorb the light being emitted from the sputtering process which occurs in the hollow cathode itself.	Use the manufacturer's recommended values for hollow cathode lamp current and spectral band width.
	Incorrect alignment of the light sources and inaccurate selection of wavelength of the monochromator	lamp must be set for maximum throughput, providing maximum energy, therefore obtaining minimum noise.
	Burner Position when flame on	burner must be carefully aligned
	Flame Stability and Flame Absorption	Before the instrument is used for an analysis, allow the hollow cathode about

		a five minute warm-up period if highly accurate measurements are required.
	Gas Pressures and Flows	he presence of liquid in the gases can yield highly irregular results. Nitrous oxide and acetylene should be sufficiently pure to prevent any impurities causing problems with the analysis
	Nebulizer and Burner	Both the nebulizer and the burner should be thoroughly clean and free from any blockages due to salts etc
	Atomic Emission	Atomic emission occurs at the same wavelength as the atomic absorption process, and the high level of light falling on the photomultiplier can result in some increase in signal noise level. In order to minimize this problem it is necessary to increase the lamp current to produce a higher source intensity, and thus to reduce the EHT applied to the photomultiplier.

REFERENCES

Roos, J. T. H. (1969). *Precision and error functions in atomic absorption spectrophotometry. SpectrochimicaActa Part B: Atomic Spectroscopy, 24(5), 255–261.* doi:10.1016/0584-8547(69)80030-3

https://www.thermofisher.com/in/en/home/industrial/spectroscopy-elemental-isotope-analysis/spectroscopy-elemental-isotope-analysis-learning-center/trace-elemental-analysis-tea-information/atomic-absorption-aa-information/aa-sample-preparation.html

https://www2.chemistry.msu.edu/courses/cem434/Chap9AtomAbsSpec.pdf

https://www.agilent.com/cs/library/troubleshootingguide/public/technicaloverview-AA-guide-5994-0858EN-agilent.pdf

https://www.agilent.com/cs/library/applications/AA012.pdf

LECTURE 10

ABSORPTION SPECTROSCOPY (INDUCTIVELY COUPLED PLASMA EMISSION SPECTROMETER): CONCEPTS AND INSTRUMENTATION

Inductively coupled plasma atomic emission spectroscopy (ICP-AES), also referred to as inductively coupled plasma optical emission spectrometry (ICP-OES), is an analytical technique used for *the detection of trace metals.* Most versatile methods of inorganic analysis followed for the past 25 years. Compared to atomic absorption spectrophotometers, in which the excitation temperature of air-acetylene flame measures 2000 to 3000 K, the excitation temperature of argon ICP is 5000 to 7000 K, which efficiently excites many elements. Also, using inert gas (argon) makes oxides and nitrides harder to be generated.

What is plasma?

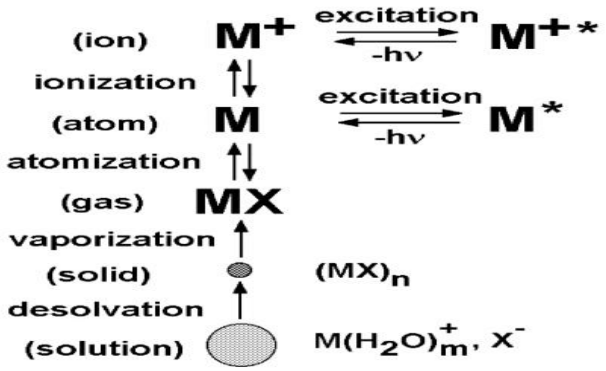
- It is an ionized gas consisting of positive ions and free electrons in proportions resulting in more or less no overall electric charge, typically at low pressures or at very high temperatures.
- It is the 4th state of matter and was first described by chemist Irving Langmuir in the 1920s
- Plasma is an electrically neutral medium of unbound positive and negative particles (i.e. the overall charge of plasma is roughly zero).
- Plasma can be artificially generated by heating a neutral gas or subjecting it to a strong electromagnetic field to the point where an ionized gaseous substance becomes increasingly electrically conductive.

Plasma in ICP

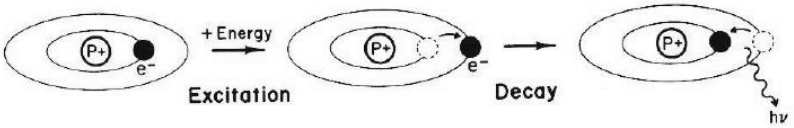
- The plasma is a gas (argon) which has been ionized inside an oscillating radio frequency field
- High voltage spark act as a initial ion generator

Principle

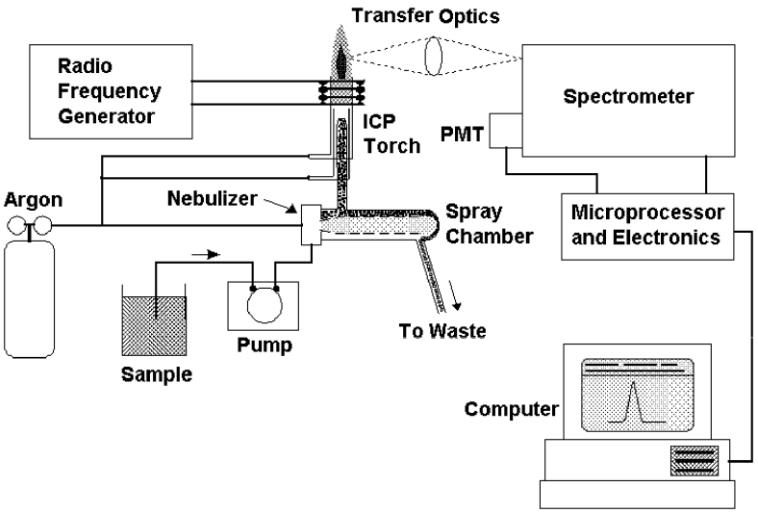
When plasma energy is given to an analysis sample from outside, the component elements (atoms) is excited. When the excited atoms return to low energy position, emission rays (spectrum rays) are released and the emission rays that correspond to the photon wavelength are measured. Element type is determined based on the position of the photon rays, and the content of each element is determined based on the rays' intensity. Ions emit electromagnetic radiation at wavelengths characteristic of a particular element. Intensity of emission is indicative of the concentration of element within the sample.



Electrons of an atom absorb energy and jump to higher energy levels. When they return to normal states, they emit characteristic photons of energy. By isolating these photon wavelengths, we can determine the types and concentrations of the elements present.



Components of ICP-OES



Sample Introduction

- Transport a sample into the central channel of the ICP as either a gas, vapor, aerosol of fine droplets, or solid particles.
- An ideal sample introduction system include amenity to samples in all phases (solid, liquid, or gas), tolerance to complex matrices, the ability to analyse very small amount of samples (<1mL or <50 mg), excellent stability and reproducibility, high transport efficiency, simplicity, and low cost.
- A wide variety of sample introduction methods have been developed, such as *nebulization*, hydride generation (HG), electro thermal vaporization (ETV), and laser ablation.

Types of Nebulizers

Pneumatic

- Use high-speed gas flows to create an aerosol

Ultrasonic nebulizers (USNs)

- Breaks liquid samples into a fine aerosol by the ultrasonic oscillations of a piezoelectric crystal & independent of the gas flow rate.
- Provide more sample delivery and improves detection
- Piezoelectric crystal rapidly vibrates to genreate sonic energy and create extremely fine droplets at low flow rates are completely transferred to the plasma

Grid type

- Create a fine mist by placing a grid in front of the argon flow.
- Liquid sample is allowed to flow down the grid and as argon passes through the grid it creates fine droplets.

Pneumatic nebulisation

- Pneumatic nebulization is very inefficient,- only a very small fraction (less than 5%) of the aspirated sample solution actually reaches the plasma. Most of the liquid is lost down the drain in the spray chamber.
- However, the pneumatic nebulizer retains its popularity owing to its *convenience, reasonable stability, and ease of use*. Efficiency may only be a concern when sample volumes are limited, or measurements must be performed at or near the detection limit.
- Three types of pneumatic nebulizers are commonly employed in ICP/OES: *the concentric nebulizer, the cross-flow nebulizer, and the Babington nebulizer.*

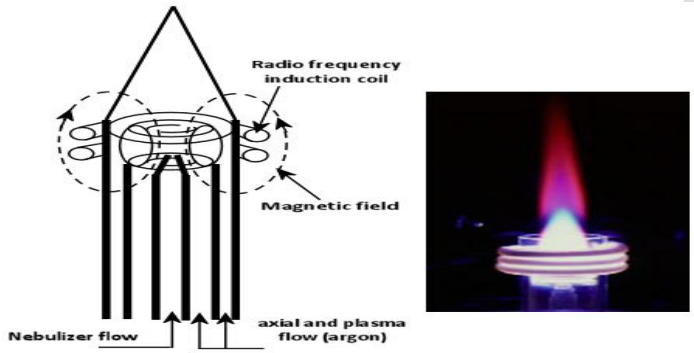
Spray Chamber

- Thermally stabilized spray chambers are sometimes adopted to decrease the amount of liquid introduced into the plasma, thus providing stability especially when organic solvents are involved.
- Sample must be transported to the torch for injection into the plasma. Hence, a *spray chamber* is placed between the nebulizer and the torch to allow small droplets of aerosols.
- *Spray chamber is to remove large droplets from the aerosol and* to smooth out pulses that occur during nebulization, often due to pumping of the solution.
- In general, spray chambers for the ICP are designed to allow droplets with diameters of about 10 mm or smaller to pass to the plasma. With typical nebulizers, this droplet range constitutes about 1 - 5% of the sample that is introduced to the nebulizer. The remaining 95-99% of the sample is drained into a waste container.

- The material from which a spray chamber is constructed can be an important. Spray chambers made from *corrosion-resistant materials* allow the analyst to introduce samples containing *hydrofluoric acid* which could damage glass spray chambers.

Torch

- Used to create and sustain a plasma.
- A plasma is an electrically conducting gaseous mixture containing enough cations and electrons (though the plasma has a neutral charge overall) to maintain the conductance.
- DC arc and microwave plasmas can also be used to generate plasmas, but for purposes of metal analysis, the inductively coupled plasma system is most important.



The purpose of the torch is to

1) evaporate the solvent (usually water) from the analyte salts, (2) atomize the atoms in the salt (break the ionic bonds and form gaseous state atoms), and (3) excite or ionize the atoms.

Torches contain three concentric tubes for argon flow and aerosol injection. Spacing between the two outer tubes is kept narrow so that the gas introduced between them emerges at high velocity.

Radio Frequency Generator

- This device that provides the power for the generation and sustainment of the plasma discharge.
- This power, typically ranging from about 700 to 1500 watts, is transferred to the plasma gas through a load coil surrounding the top of the torch.
- The load coil, which acts as an antenna to transfer the RF power to the plasma, is usually made from copper tubing and is cooled by water or gas during operation.
- There are two general types of RF generators used in ICP instruments.
- Crystal-controlled generators use a piezoelectric quartz crystal to produce an RF oscillating signal that is amplified by the generator before it is applied at the load coil.

Separation and Viewing

Spectrometers are used to isolate the emitted spectral lines of analyte from the all other emissions. Following are the available devices for this purpose.

- Monochromators : single spectra

- Polychromators : paschen-runge optics coupled to highly sensitivity detectors known as photomultiplers (PMT)
- Echelle grating optics: coupled to solid state detectors (CCD,CID) also known as charge transfer devices.

Based on position of the monochromator entrance relative to the plasma source, two choices are available viz., radial and axial alignment.

Radial	Axial
Allows photons to move from side of plasma (at the end of the torch) to the entrance slit	Gives higher intensities (and better detection limits) since photons come from center and end of plasma.
Useful for samples with higher concentrations; avoids burning out the detector, which is costly to replace.	use the axial monochromator alignment for lower concentration samples.

Categories of detection

Broad Categories are available for analyzing the emitted photons:

- Sequential,
- Simultaneous multi-channel, and
- Fourier transforms systems.

In the first two, all wavelengths enter a monochromatic where they are dispersed by prisms and/or grating monochromators and are then transmitted to the detector (most commonly the PMT or CCD/CID

In Fourier transform systems, no slits or monochromators are required, and this creates better detector limits because more intense radiation reaches the detector. Fourier transform systems also have higher spectral resolution (and thus have fewer spectral interferences) and can simultaneous monitor all wavelengths for longer times.

SAC 454

LECTURE 11

ABSORPTION SPECTROSCOPY (INDUCTIVELY COUPLED PLASMA EMISSION SPECTROMETER): PREPARATION OF SAMPLES AND STANDARDS, INTERFERENCES, TROUBLE SHOOTING AND MAINTENANCE

Sample Preparation for ICP-OES analysis

A variety of sample types can be analyzed by inductively coupled plasma optical emission spectroscopy (ICP-OES), including aqueous and organic liquid and solid samples. These have to be brought into a state that the ICP and the instrument as a whole can process for elemental analysis. The most typical sample form is a liquid. A liquid sample is introduced using a peristaltic pump to ensure constant flow. Commonly, a high-speed flow of gas (usually argon) is used to shatter small droplets of liquid into an aerosol. This aerosol is then introduced into a spray chamber which removes the larger droplets. Only the fine aerosol is allowed to enter the plasma. Solid samples are typically ablated into small particles either using a laser or spark ablation system or then transported directly to the plasma.

Aqueous samples

Aqueous samples can be introduced into a plasma directly, and often without dilution. They are typically acidified with nitric acid (HNO_3) to ensure that their elemental components remain in solution. A standard sample introduction system usually consists of a concentric nebulizer and a cyclonic spray chamber. With regards to particles in solution, a standard nebulizer can handle particle sizes of up to one third of the capillary diameter.

Solid sample digestion

Because solid samples cannot be introduced into the plasma directly, they must be either transferred into the plasma using an accessory technique (e.g., electrothermal vaporization, laser ablation), or they must be made into a solution. The main techniques used to dissolve solids are acid digestion and fusion.

While using fusion for sample preparation leads to complete sample dissolution, it also results in high solid content, which increases the risk of contamination from flux agents. As a result, sample dilution is needed and this reduces the detection limit.

Acid digestion usually involves the dissolution of a sample in a hot acid, or a mixture of hot acids. Heating acids is performed on a hot plate, or by using a microwave digestion system that employs pressurized vessels to produce even higher reaction temperatures.

Acid digestion of soils and sludges is one example of solid sample preparation for ICP-OES. The digestion or extraction of soil samples is performed with aqua regia, which is a solution composed of

nitric and hydrochloric acid at a 1:3 ratio. This solution is capable of releasing most elements from the soil.

Special digestions

If the soil (or other sample types like plant material) contains high concentrations of silicon dioxide, then hydrofluoric acid (HF) is added to the digestion protocol. HF is not only highly corrosive and toxic: it also dissolves glassware used in the process of sample preparation and introduction into the ICP-OES. In such cases, a special sample introduction system, which is inert to HF, must be used.

For samples that contain high amounts of organic material, (e.g., food), hydrogen peroxide is added during digestion because it accelerates the dissolution process by raising the sample core temperature. For the digestion of plastic material, a strong oxidizing agent such as sulfuric acid is applied; however, many resulting sulfates are insoluble.

Standard Preparation

- Ensure purchased standards are still within "Use By" date
- Use calibrated pipettes and class 'A' volumetric flasks for dilutions
- Periodically, check accuracy & reproducibility of your pipettes
- Use de-ionized water (Type I - conductivity /cm3δ> 18 M)
- Use serial dilutions for preparing low concentrations from 10,000 ppm stock
- Please don't do large dilutions (> 1:10,000) in 1 step
- Prepare ppb (ug/L) concentration standards daily from high conc. stock
- Prepare low ppm (mg/L) concentration standards weekly
- Store in plastic vessels for ensure better stability
- Stabilize with acid – low pH ensures better stability

COMMON PROBLEMS IN ICP-AES / INTERFERENCES

- Sampling and Sample Preparation
- Spectral Interference
- Matrix Effects
- Instrumental Drift

Spectral Interference

- Some elemental lines may interfere with others.
- Best solution is to find another spectral line.
- Samples should be scanned for possible problems

Matrix Effects

- Differing viscosities can affect amount of sample uptake

- Matrices can change nature of plasma
- Certain matrices (HF) can attack torch
- Matrices can contain interfering spectral components

Matrix effect

Instrumental Drift

- Instrument reading can drift over a period of time due to *physical changes in the optical system, or the configuration of the plasma.*
- Standards need to be run at the beginning and end of each run in order to estimate and correct for this drift.
- Internal standards are used to compensate for differing matrices from sample to sample.

Compensation

- Standards run with every sample run
- Drift Correction or internal standardization is taken with every sample run
- Matrix of standards should be closely matched with that of the samples
- Preliminary scans are taken to see if any spectral overlaps occur

MAINTENANCE

- Check exhausts system operating (smoke test)
- Inspect torch for injector blockage/other damage
- Check nebulizer for blockage/pulsation
- Inspect peristaltic pump tubing for stretching or flatness

After analysis is complete:

- Aspirate rinse solution for 5-10 mins before shutting down (minimizes sample deposits)
- Release pressure bar and detach peristaltic pump tubes from holder
- Empty waste vessel
- Leave ICP-OES in stand-by mode (gas and power on; software shutdown)

TROUBLESHOOTING

S.No.	Problem	Rectification
1.	Poor calibration	• Plasma should be warmed up and stable • Optics boost purge should be enabled and stable

SAC 454

		• Check that tuning solution had reached plasma - Check that purge enabled and stable – then repeat
2.	Plasma Ignition Problems	• Check the grade of argon • Check all connections in the sample introduction system for cracks, loose fittings, missing or damaged items • Check that the plasma and auxiliary gas connections to torch were not reversed • Check that the torch is sitting correctly w.r.t. RF coils (top of intermediate tube should be 2-3mm from edge)
3.	Memory Effects	• Typically observed when measuring high concentrations of selected analytes • Usually see high intensity for first replicate – subsequent replicates are more consistent • Check the rinse time used is adequate (30-40s is typical) • Use an acidified (matrix matched) rinse solution • Switch to a spray chamber that has smaller internal volume • Use a "switching valve" to improve washout characteristics
4.	Potential Autosampler Issues	• Confirm samples loaded in correct sample locations • • Long transfer tube between sampler and ICP-OES • Ensure probe diameter is appropriate for sample matrix - Use wider bore for high % TDS or viscous samples • Sample stability - potential for sample changes while uncovered in racks • Ensure transfer line to ICP-OES is in good condition

APPLICATIONS OF ICP-AES

- **Clinical analysis**: metals in biological fluids (blood, urine)
- **Environmental analysis**: trace metals and other elements in waters, soils, plants, compost and sledges.
- **Pharmaceuticals**: traces of catalysts used; traces of poison metals (Cd, Pb etc.,)
- **Industry**: trace metals analysis in raw materials; noble metals determination
- **Forensic science**: gunshot powder residue analysis, toxicological examination (e.g., thallium (Tl) determination.

SAC 454

COMPARISON OF FLAME PHOTOMETRY, ICP-AES AND AAS

Particulars	**Flame photometry**	**ICP-AES**	**Atomic absorption spectroscopy**
Principle	Emission	Emission	Absorption
Instrumentation	No light source	No Light source	Light source (Hallow cathode lamp)
	Burner used	Not used	Laminar flow Burner used
Heat source	Flame	Plasma	Flame
Process	Excitation	Excitation	Ground state(light absorbed by unexcited atoms)
Law	Beer's law Not applicable	Beer's law Not applicable	Beer's law
Data obtained	I Vs C	I Vs C	A Vs C
Uses	Alkali and alkali earth metals	Mostly all elements in periodic table except few.	Micro-nutrients

REFERENCES

- https://www.agilent.com/cs/library/eseminars/public/Atomic_Presentation_ICP-OES_Maintenance_Updated_Oct_2016.pdf

SAC 454

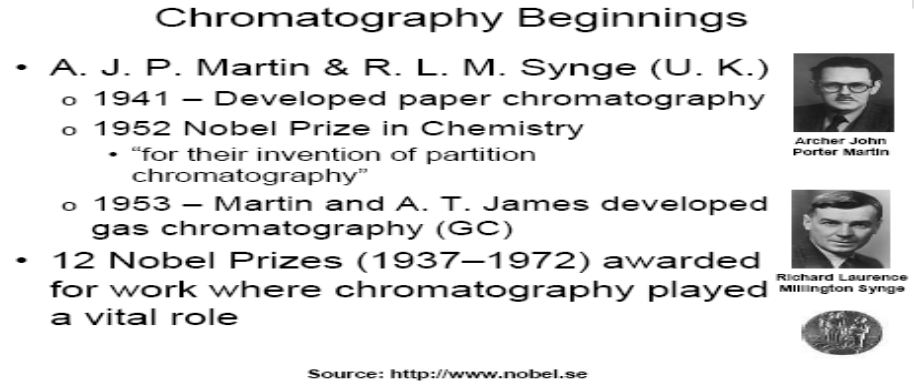
LECTURE 12

PRINCIPLE AND PRACTICE OF PAPER CHROMATOGRAPHY, THIN LAYER CHROMATOGRAPHY

Chromatography basically involves the separation of mixtures due to differences in the distribution coefficient (equilibrium distribution) of sample components between 2 different phases. One of these phases is a mobile phase and the other is a stationary phase.

Chromatography Beginnings

- Mikhail Tswett (Russian botanist) 1903 – Separated various plant pigments by passing solutions through glass column packed with finely divided $CaCO_3$
- Separated species appeared as colored bands on the column
- Chose the name "chromatography" for this technique
- Chroma = "color"
- Graphein = "write"



Principle of chromatography

- Chromatographic separation involves the placing of a sample onto a liquid or solid (stationary phase) and passing a liquid or gaseous (mobile phase) through or over it, a process known as elution.
- Sample components or solutes, whose distribution ratios between the two phases differ, will migrate at different rates, and this differential rate of migration will lead to their separation over a period of time and distance.

Table 1. A classification of the principal chromatographic techniques

Technique	Stationary phase	Mobile phase	Format	Principal sorption mechanism
Paper chromatography (PC)	Paper (cellulose)	Liquid	Planar	Partition (adsorption, ion-exchange, exclusion)
Thin-layer chromatography (TLC)	Silica, cellulose, ion-exchange resin, controlled porosity solid	Liquid	Planar	Adsorption (partition, ion-exchange, exclusion)
Gas chromatography (GC)				
Gas-liquid chromatography (GLC)	Liquid	Gas	Column	Partition
Gas-solid chromatography (GSC)	Solid	Gas	Column	Adsorption
Liquid chromatography (LC)				
High-performance liquid chromatography (HPLC)	Solid or bonded-phase	Liquid	Column	Modified partition (adsorption)
Size-exclusion chromatography (SEC)	Controlled porosity solid	Liquid	Column	Exclusion
Ion-exchange chromatography (IEC) Ion chromatography (IC)	Ion-exchange resin or bonded-phase	Liquid	Column	Ion-exchange
Chiral chromatography (CC)	Solid chiral selector	Liquid	Column	Selective adsorption

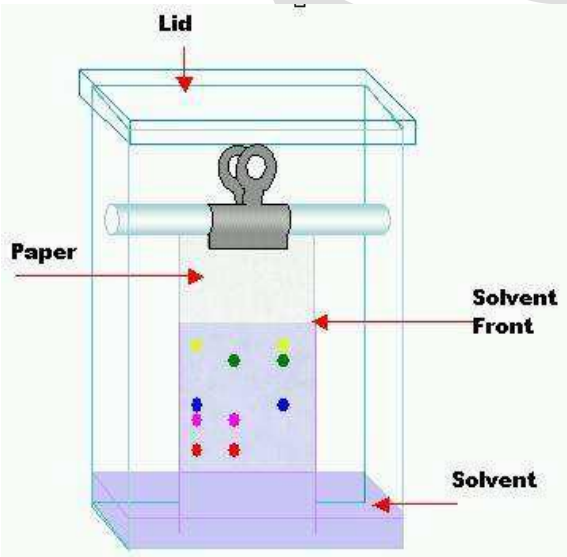
Planar chromatography

- Planar chromatography includes thin-layer and paper chromatography and electrophoresis.
- In thin-layer chromatography (TLC), a stationary phase is coated on an inert plate of glass, plastic, or metal.
- The samples are spotted or placed as streaks on the plate.
- Development of the chromatogram takes place as the mobile phase percolates through the stationary phase and the spot locations.
- The sample travels across the plate in the mobile phase, propelled by capillary action.
- Separation of components occurs through adsorption, partition, exclusion, or ion-exchange processes, or a combination of these
- Paper chromatography (PC) is simple and cheap but lacks the separating power and is an analytical method that is used to separate colored chemicals or substances, especially pigments.
- This can also be used in secondary or primary colours in ink experiments.
- This method has been largely replaced by thin layer **chromatography**, but is still a powerful teaching tool.

Both require only inexpensive equipment and reagents, and comparisons can be made between a number of samples and standards simultaneously

PRINCIPLE OF PAPER CHROMATOGRAPHY

- In paper chromatography the substrate is a piece of porous paper with water adsorbed on it.
- The sample is placed on the paper as a spot or streak and then irrigated by the solvent system that percolates within the porous structure of the paper.
- Usually development of the chromatogram is stopped before the mobile phase reaches the farther edge of the paper, so the solute zones are distributed in space instead of time.
- The major limitations of paper chromatography are relatively long development times and less sharply defined zones as compared to thin-layer techniques.
- Thin-layer chromatography with powdered cellulose as stationary phase has displaced paper chromatography from many of its previous applications
- Solvent Systems: Chromatography on paper is essentially a liquid–liquid partition in which the paper serves as carrier for the solvent system.
- Aqueous systems are used for strongly polar or ionic solutes.
- Water is held stationary on the paper as a "water–cellulose complex", organized and dense near the amorphous regions of the cellulose chains.
- The stationary phase is attained by exposing the suspended paper to an atmosphere saturated with water vapor in a closed chamber.
- If an aqueous buffer or salt solution is to be used as the stationary phase, the paper is drawn through the solution, allowed to dry, and then exposed to the atmosphere saturated with water vapor



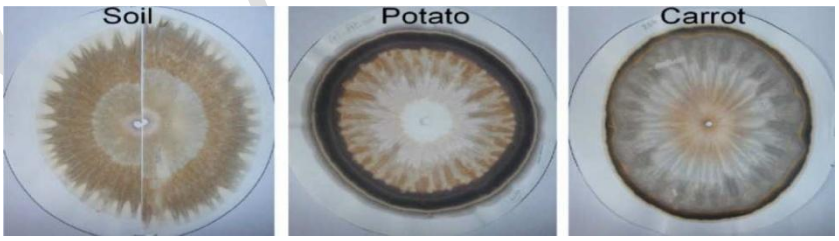
- ☐ The mobile phase might be butanol for a neutral system, butanol–acetic acid– water (40:10:50) for an acidic system, or butanol–ammonia–water (75:8:17) for a basic system.

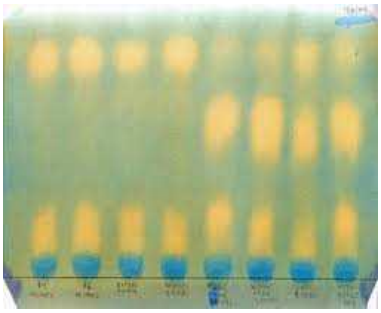
- The latter two systems are prepared by shaking all components in a separator funnel; the less polar phase serves for development. Even water itself can serve as developer
- Equipment: The only essential piece of equipment is the developing chamber, often simply an For one-dimensional paper chroenclosed container with an airtight lid.matography, either ascending or descending development can be carried out in simple units.
- Descending development is more often used because it is faster and more suitable for long paper sheets, which give higher efficiencies.
- Radial development is carried out by cutting a tab from a circular piece of paper, spotting the sample at the upper end of the table or streaked in a circle a short distance from the center of the paper, and then placing the tab in the solvent reservoir.
- Small chambers are made from two petridishes, one inverted over the other.

Development:

- Each of the development techniques described for thin-layer chromatography is equally applicable for paper chromatography. One difference should be noted.
- The paper is equilibrated with both mobile and stationary phases for 1 to 3 h before development.
- The two phases are placed in separate reservoirs in the bottom of the chamber.
- The mobile phase should also be equilibrated with the stationary phase.
- Close temperature control is required for reproducible Rf values.
- With volatile solvents, careful equilibration is required to avoid band tailing

Visualization and Evaluation of Paper Chromatograms:

- As done in TLC, interpretation methods are applicable for paper chromatography with the exception of fluorescent quenching using TLC plates impregnated with a phosphor
- 



SAC 454

- Special attachments for commercial transmission spectrophotometers provide a means for drawing the paper chromatogram across a window in front of a photo detector.
- There flection mode can also be used. An assessment of the area under the photometric curve completes the measurement.

TYPES OF PAPER CHROMATOGRAPHY

1. Descending Paper Chromatography

Development of the chromatogram is done by allowing the solvent to travel down the paper is called Descendiing

2. Ascending Paper Chromatography-

Solvent travel upward direction of the Chromatographic paper.

Both the Descending and Ascending Paper Chromatography are used for separation of Organic and Inorganic substances

Ascending-Descending Paper Chromatography-

- Hybrid of both the above techniques. The upper part of the Ascending chromatography can be folded over a rod and allowing the paper to become descending after crossing the rod.

4. Radial Paper Chromatography

- It is also called a Circular chromatography.
- Here a circular filter paper is taken and the sample is given at the center of the paper.
- After drying the spot the filter paper is tied horizontally on a Petridish containing solvent. So that Wick of the paper is dipped inside the solvent.
- The solvent rises through the wick and the component get separated in form of concentrate circular zone.
- **Two-Dimensional Paper Chromatography**
- In this technique a square or rectangular paper is used. Here the sample is applied to one of the corners and development is performed at right angle to the direction of first run.

THIN LAYER CHROMATOGRAPHY

Thin-layer chromatography is a form of planar chromatography similar to paper chromatography, but the stationary phase is a finely-divided sorbent spread as a thin layer on a supporting flat plastic, aluminum or glass plate.

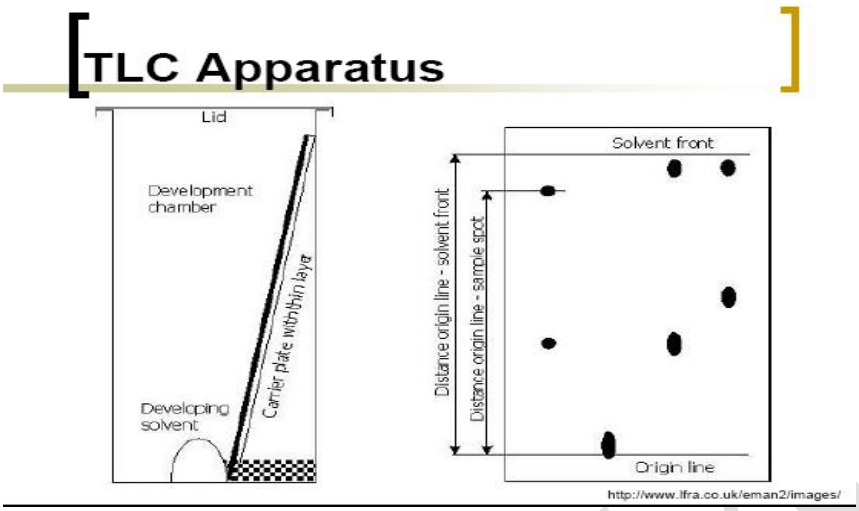
Solutes migrate through the stationary phase at rates determined by their distribution ratios, those with the largest values moving the least, whilst those with the smallest values moving with the advancing mobile phase, or solvent front.

TLC is used mainly for confirmation of residues following initial screening and quantification. Confirmation by TLC, which is based on comparison of migration distances of the herbicide of interest with authentic standards run on the same layer. In addition, the quantification can be carried out by TLC if a gas chromatograph is not available or if the herbicide of interest is unstable during determination. Extraction, cleanup and concentration steps normally precede TLC determination. Spots are applied to the thin layers using simple dispersible capillaries, GC syringes or automatic multiple spotting devices, standard solutions must be spotted on the same plate as the sample, preferably on both sides of the sample spot.

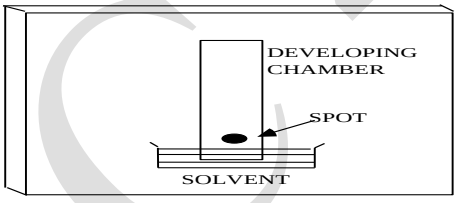
A typical TLC procedure consists of the following steps:

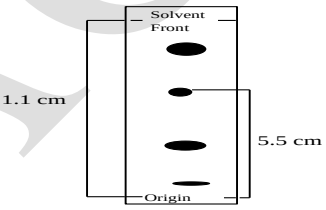
- Sufficient mobile phase to provide about a 0.5 cm depth of liquid is poured into a development tank, or chamber, which is then covered and allowed to stand for several minutes to allow the atmosphere in the tank to become saturated with the solvent vapor;
- Small volumes of liquid samples and standards, or solutions, are spotted onto the sorbent surface of a TLC plate along a line close to and parallel with one edge (the origin).
- The plate is then positioned in the tank with this edge in contact with the mobile phase and the cover replaced;
- The mobile phase is drawn through the bed of sorbent from the edge of the plate, principally by capillary action, and this development process is halted shortly before the solvent front reaches the opposite side of the plate.
- Sample components and standards migrate in parallel paths in the direction of flow of the mobile phase, separating into discrete zones or spots;
- The plate is removed from the development tank, dried in a current of warm air, and
- solute spots located by appropriate methods;

SAC 454



- Each solute is characterized by the distance migrated relative to the solvent front, i.eits Rf value, which will lie between 0 and 1, and unknowns are identified by comparisons with standards run simultaneously.
- Solvents should be of the highest purity as TLC is a very sensitive analytical technique;
- Mobile phase eluting power should be adjusted so that solute Rf values fall between 0.2 and 0.8 so as to maximize resolution





$$Rf = \frac{\text{Distance from starting origin to center of zone}}{\text{Distance from starting origin to solvent front}}$$

$$= \frac{5.5}{11} = 0.5$$

Layers are hand coated with a commercial adjustable spreading device or layers pre-coated on glass, plastic or aluminium backing may be used. Analytical layers are usually 250µ thick. Pre-coated layers are of high purity and uniformity and are used almost exclusively in most laboratories, especially for *insitu* quantification by densitometry. Activation of adsorbent layers (e.g. 80 – 110 ° C for 30 - 60 minutes) prior to spotting and development is often required. Once the adsorbent has been activated, it must either be used promptly or stored under desiccating conditions or activation must be repeated. Silica gel and alumina layers usually give the best results, but polyamide, microcrystalline cellulose,

ketselguhr, zinc carbonate and magnesium oxide are among other adsorbents have also been used. Chromatography is carried out, in a development chamber, most often a rectangular glass paper-lined tank saturated with solvent vapors. Ascending development for a distance of 10-20 cm is typical. It is important to follow exactly all stated conditions when attempting to reproduce a separation. The temperature, development chamber design and equilibration and water content of the adsorbent are probably the most frequent sources of variation among laboratories.

The technique termed 'High Performance Thin Layer Chromatography' (HPTLC) has become increasingly important for separations and *insitu* quantitative analysis in the recent past. HPTLC is carried out on 10 x l0 cm or 5x5cm layers of silica gel with a smaller particle size and a narrower particle size distribution than in conventional TLC plates and thereby gives improved resolution and sensitivity of detection. Volumes not larger than 1 µl must be spotted to realise best results. For manual application, spotting is usually done with a pt-Fe tipped nano pipette or equivalent or this type of pipette is used with an automatic spotting device that control both the pressure of the pipette tip on the layer and the duration of contact. Solvent development is carried out in the miniature glass rectangular chamber. High resolution is achieved rapidly with short development distances.

The following solvent systems have provided to be generally useful for separation of a wide range of herbicides on silica gel thin layers; benzene mixed with varying amounts of ethanol for polar, and a mixture of hydrocarbon plus acetone plus chloroform, with the addition of methanol for non polar herbicides. Examples include pentane-acetone-chloroform (65:30:5 v/v) or pentane-acetone-methanol-chloroform (70:15:10:5 v/v). Proportions of the components are changed to suit the requirements of specific separations.

After development and air-drying of the layer, spot detection may be achieved in number of ways. Few herbicides are naturally colored but colored derivatives may be made prior to spotting, e.g. dyes from aromatic amine moieties of urea herbicides by coupling with N-ethyl- I -napthylamine. Colourless spots can be detected by spraying a chromogenic reagent with a commercial aerosol spray. Fluorescent spots can be detected under short (254 nm) or long (366 nm) wave UV light or fluorescence may be induced by application of fluorogenic reagents after development of preparation of fluorescent derivatives prior to spotting. Spots that absorb UV light are detected as quenched (dark) spots on layers containing phosphorus activated by UV light (usually 254 run). Radio active (labeled) herbicides are detected by autoradiography and by direct bio autography.

Quantification of separated spots may be achieved by "eye ball" comparison between sample and standard spots run on the same plate or by some independent analytical method (spectrophotometry or GC) after scraping the spot, collection and eluting the herbicide from the absorbent. Manual elution is

simply carried out by scraping in a vial or tube adding solvent and agitating (vortexing), filtering the adsorbent, and concentrating the filtrate containing the herbicide. An automated elution system is also available in modern equipments. Radioactive spots can be quantitated by scintillation counting after scraping or by automatic scanning of radioactivity on the layer.

Colour, fluorescent or quenched spots may be scanned on the layer when a spectro densitometer is available. Quantificationis achieved by scanning sample and standard spots in the optimum instrumental mode and treating the resultant peaks representing the amount of light absorbed or emitted in the same manner as GC peaks for calculations. Important considerations for densitometry are precise and accurate spotting uniform layers, RF values between 0.3 and 0.7, an application of detection reagents, and optimum use of a good densitometer.

Advantages of TLC

- Simplicity, rapidity and low cost
- Sensitivity ranges from about 5-500 ng for most pesticides detection methods. Semi quantitative estimation
- Visual comparison of sample and standard spot sizes and or intensities
- More accurate quantification
- Insitu scanning of spots with a spectro densitometer

SAC 454

LECTURE 13

GAS CHROMATOGRAPHY: PRINCIPLE, TYPES AND INSTRUMENTATION

Chromatography has been developed as a new method of separations of mixture of substances especially when they are available in small amounts. The important instrumental methods of chromatography are

- Gas (GC) and high performance liquid chromatography (HPLC) are complementary techniques best suited to the separation of volatile and nonvolatile mixtures, respectively.
- Both these techniques are instrument based and computer controlled, with sophisticated software packages and the ability to separate very complex mixtures of up to 100 or more components.

Key terms commonly used in chromatography analysis.

Chromatography: Separation technique

Chromatograph: Separation equipment

Chromatogram: Out-put chart obtained from the analysis.

Eluent: The mobile phase

Elution: Motion of the mobile phase through the stationary phase.

Eluate: Fluid exiting the column

Resolution: degree of separation of different solutes. Resolution can be improved by using a longer stationary phase, finer stationary phase or slower elution.

Retention Time : Time required for the sample to travel from the injection port through the column to the detector.

Gas Chromatography

Gas chromatography is an instrumental method for the separation and identification of chemical compounds. In pesticide analysis, GC has assumed a role of prime importance as compared to other methods. This is because GC technique is capable of rapidly resolving complex herbicide mixtures and provides qualitative identification and precise quantitative analysis of the compounds. In multi-residue analysis, GC is the only method of choice. The GC unit consists basically of the following elements.

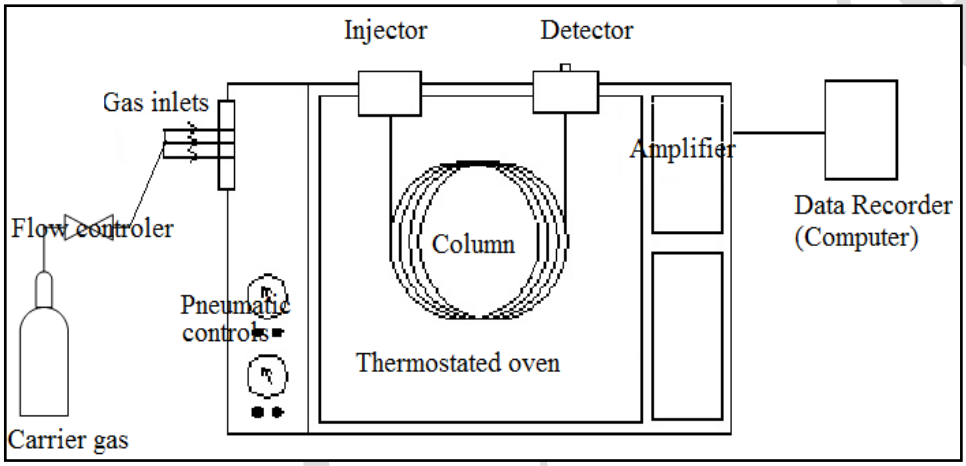
Carrier gas system: The carrier gas ensures the migration of components of sample to be separated. The nature of the carrier gas must be non reactive with the stationary phase or the sample components. The gases like nitrogen argon and helium are widely used.

SAC 454

Injection port: The injector system is to introduce the gas, liquid or solid sample rapidly and in a reproducible manner in to the column. Syringes of 1, 2 and 5 µl volumes are most commonly used in gas chromatography and with 1 µl syringe, 0.01 µl can be injected directly.

Chromatographic column: With respect to their dimensions and arrangement of two basic types of columns, the packed and the open tubular (capillary) column are rather different, though the latest type (packed capillary columns) represents a certain transition between the two.

Oven: The oven is made of metal block in which the GC column is enclosed. The temperatures of injection block, column, oven and detector can be varied and controlled. Injection block temperature is generally 20 to 50^{0C} higher than the column temperature.



Schematic Diagram of Gas Chromatography

Detectors: Detectors have the task to sense continually, rapidly the components which appear in the carrier gas as it emerges from the column with high sensitivity. The detector senses the changes in certain physical or chemical property of the eluent gas stream on the appearance of the components. With the help of an appropriate current source and signal transmitted, the detector gives a direct electric signal or the response of the detector is converted into an electric signal by an appropriate electronic system. Usually this electrical signal is a voltage, which may be led directly or through an amplifier for recording or data processing. A good detector should possess:

- ☞ High sensitivity (in some cases selectivity) towards the various components.
- ☞ The response should follow rapidly the actual change.
- ☞ Should give in a wide concentration range, signals proportional to the quantity of the component (linearity).
- ☞ Low sensitivity to fluctuations in operation conditions (carrier gas rate, pressure and temperature).
- ☞ Stable operation with respect to both noise and drift.

In addition to the above basic operation aspects, it is important that the detector should be easy to build, cheap of rugged construction, easily operated, safe from dangers of explosion or radiation and should, if possible not require expensive and complicated auxiliary fixtures.

GC detectors, working principle and type of compounds detected

Detectors	**Working principle**	**Application**	**Sensitivity**
Thermal Conductivity Detector (TCD)	heat conduction by gas (dependent on gas molecular weight)	All gases detected. Universal detector	Variable sensitivity. Not very sensitive
Flame Ionization Detector (FID)	GC effluent burned in hydrogen flame producing ions measured by electrode	Hydrocarbons are detected ;reduced response for other functional groups	Good sensitivity Quantitation is good and possible without standards
Electron Capture Detector (ECD)	Uses β emitter to produce electrons that cause current	Selective for compounds with electronegative groups (e.g. halogens, nitro groups, etc.)	Very sensitive detector
Photo ionization Detector (PID)	Uses UV light to photo ionize compounds (M + hv → M+ + e-)	Element Specific Detectors (common for S, P, and N)	Sensitive to unsaturated compounds
Mass Spectrometry.	Detects and measures by converting eluting analytes into gas phase ions (forming a molecular ion or fragmenting analyte	Tunable for compounds	Multi residue analysis. Compare patterns of ions and fragments to known values.

Performance of GC detectors

Detector	**Element or functional group**	**Detection limit(pg/sec)**	**Herbicide analysis application**
ECD	Trichloro nitro	0.50	2,4-D and 2.4.5-T esters, polynuclear aromatics, nitro herbicides
	Polyhalo- genated	0.05	OC compounds, pentachlorophenol, dioxins

FPD	P	0.20	OP compounds,
	S	4.00	Carbamates containing S, ethylene thiourea
AFID/ TID	P	0.02	OP compounds
	N	0.20	Carbamate insecticides, triazines, urea and nitro herbicides, nitrosamines
HECD	Cl	0.50	Phenoxy herbicides
Hall -EC	N	1.00	Carbamate insecticides, triazines, urea and nitro herbicides, nitrosamines

Recorders and Integrators: The response of the detector is recorded using computer enabled software's in the form of chromatogram. Measurement of peak areas and retention time with high accuracy, correction for base line drift and analysis of partially resolved peaks by a curve fitting procedure to obtain percentage of each component are some of the operations that are carried out by computers.

INSTRUMENTATION

- When the system is ready, as indicated by the ready light, samples are injected into the injector port where they are vaporized and carried into the column by the carrier gas.
- Separation of the components of the mixture occurs in the column.
- Compounds differentially retained in the stationary phase reach the detector at different times to produce a set of peaks along the time line.
- The detector response is sent to a computer system where the progress of the sample is monitored on the computer monitor in graphical form that displays detector response as a function of run time.
- Each component of the mixture reaches the detector at a different time and produces a signal at a characteristic time called a retention time.
- The area under a peak is related to the amount of that component present in the mixture.
- The detector information can also sent to a printer that produces hard copy of the chromatographic run.

SAC 454

Quantitative determination of residues

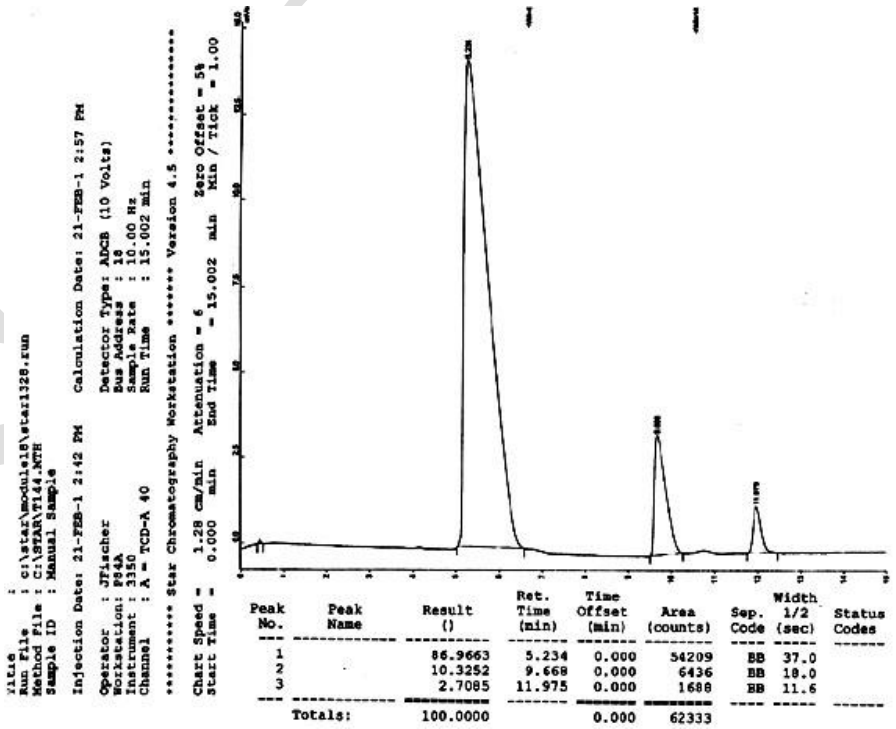
GC is the most powerful analytical tool for quantitative analysis and is capable of giving accurate results. A typical chromatogram is enclosed to calculate the residue content in the samples.

$$\text{Residue (in ppm)} = \left(\frac{\text{Peak height of thesample}}{\text{Peakheight of the standard}}\right) \text{x} \left(\frac{\text{Concentration of corresponding std}}{\text{Weight of sample}}\right) \text{x Dilution}$$

- Due to its high efficiency, GC allows the separation of the components of complex mixtures in a reasonable time.
- Accurate quantitation (usually sharp reproducible peaks are obtained)
- Mature technique with many applications notes available for users.
- Multiple detectors with high sensitivity (ppb) are available, which can also be used in series with a mass spectrometer since MS is a non-destructive technique.

Disadvantages of GC

- Limited to thermally stable and volatile compounds.
- Most GC detectors are destructive, except for MS.



Sample chromatogram

LECTURE 14

GAS CHROMATOGRAPHY: OPERATION, SAMPLE HANDLING, MAINTENANCE AND TROUBLE SHOOTING AND APPLICATIONS

GC OPERATION

For operating a GC, the following steps should be followed.

1. **Type of application.** While gas chromatography remains the same at the core, different applications require different accessories (columns, injectors and detectors) to run the machine. Hence based on the analyte nature, type of sample, analyte chemical property, choose the column, detector, injector and carrier gas. Choosing an appropriate calibration gas for the application is essential as it is used to provide the basis to measure the retention time of analyte.
2. **Check accessories and turn on the machine:** Ensure that the accessories are properly attached to the machine and have no leaks. Once the machine is turned on, it will perform preliminary checks.
3. **Set up instrument method in the software.** This is a set of commands created that the chromatograph follows. These commands include settings such as: temperature ramp profile, final temperature, and valve duration.
4. **Perform a blank run.** A blank run is a way the chromatograph clears itself from residual components left unintentionally from previous runs. This is crucial to ensure that the test sample is not contaminated by residual components. Blank runs do not need a sample injected as they rely on the carrier gas to move the residual components out of the machine. Blank runs can be created by the user using an instrument method as explained in the previous step. Perform a couple of blank runs to clean the system completely.
5. **Inject a sample of the calibration gas into the machine.** Run the same case multiple times in order to account for errors that change the retention times and differences in peak height. Run multiple calibration gas samples with different concentrations of the desired components to form a complete calibration curve. Don't forget to run blank runs between your calibration cases. This will increase the accuracy of the calibration.
6. **Enter calibration concentrations using the available software**. Once all of calibration runs are complete, create a new calibration standard. A calibration standard is a reference used by the software to quantify the amount of components of unknown gas samples.
7. **Perform another blank run after calibration and before operating the chromatograph:** Remember, the goal is to avoid contamination that might skew the results produced by the gas chromatograph.
8. **Inject sample into the machine with the specified instrument method created earlier:** The chromatograph should display the concentrations of the species within the sample if calibrated correctly.

SAMPLE HANDLING

The commonly used techniques for the extraction of analyte from agricultural samples are detailed below.

s.no.	Method of Sample Pre-treatment	Principles of Technique
1.	Solid-Liquid Extraction	Solvent is added to dissolves/extracts/leaches the analyte of interest; solution is separated from solid by filtration (sometimes called "shake/filter" method)
2.	Soxhlet Extraction	Constantly refluxing fresh solvent flows through the sample in thimble and dissolves analytes that are continuously collected in a boiling flask. Soxhlet extraction is a classical method which is operated under atmospheric pressure, in high temperature or under ultrasonic irradiation. In this technique, relatively large volumes of organic solvents are usually used, and it is a time-consuming technique
3.	Sonication	Ultrasonic extraction, also known as sonication, uses ultrasonic vibration to ensure intimate contact between the sample and the solvent. Sonication is relatively fast, but the extraction efficiency is not as high as some other techniques. Also, ultrasonic irradiation may decompose some of organophosphorus compounds.
4.	Solid Phase Extraction (**SPE**)	Sample is applied to, and liquid is passed through, a column packed solid phase that selectively removes analyte or interferences; analyte can be eluted with strong solvent.
5.	Liquid-Liquid Extraction	Sample is partitioned between two immiscible phases which are chosen to maximize differences in solubility; interference-free analytes are then recovered from one of the two phases.
6.	Solid-phase microextraction (SPME)	It is a modern technique that consists in direct extraction of the analytes with the use of a small-diameter fused silica fiber coated with an adequate polymeric stationary phase.

SAC 454

7.	Supercritical fluid extraction **(SFE)**	In supercritical fluid extraction (SFE), supercritical fluids possess specific properties which make them facilitate the extraction of organics from solid samples.
8.	Accelerated solvent extraction **(ASE)**	accelerated solvent extraction (ASE) are pressurized fluid extraction (PFE) and pressurized liquid extraction (PLE). Conventional solvents are used in ASE at high temperature (100–180°C) and pressure (1500–2000 psi) to increase the extraction percentage of organic compounds from solid samples.
9.	**Microwave-assisted extraction**	Microwave-assisted extraction (MAE) is different from microwave-assisted acid digestion. MAE is applied for the extraction of semivolatile and nonvolatile compounds from solid samples.
10.	**Headspace extraction**	the sample can be placed directly into the headspace vial and the volatile analyte released can be injected directly into a GC column and analyzed with no additional preparation procedures.

Either single or combinations of the above methods are used to extract the analyte from sample matrix. Solid phase extraction is one of the more widely used sample preparation techniques for liquid samples or solid samples that have been put into a liquid form by dissolution or extraction. SPE can also be used for certain gaseous sample by trapping them on a sorbent or by in situ derivatization using reactive chemicals.

MAINTENANCE AND TROUBLE SHOOTING

SAC 454

Table 1

Problem	Manifestation	Probe Attributes (to include in a reference standard)	Test/Metric
Activity	Lost peaks, lower peak areas, degraded peak shapes due to decomposition or adsorption - leading to under reporting of quantity, false negatives, lower accuracy and repeatability	Compound(s) with lability and functionality to target compounds, or the target compounds themselves.	Using concentrations near the method detection limits is the most stringent test. Measure responses and peak shapes (e.g., tailing factor) relative to reference results.
Column degradation: retention and selectivity changes	Lower column efficiency leading to degraded resolution. Changes in selectivity, degraded separation, changing retention times, peaks falling out of retention time windows.	Closely eluting pair(s) of peaks with largely differing polarities (sensitive to selectivity change). At least one inert probe (like saturated hydrocarbon).	Column efficiency calculation on inert probe(s), retention times relative to reference method (most telling to do both under isothermal conditions). Resolution between peak pairs.
Column degradation: contamination and stationary phase degradation	Increased background, ghost peaks, degraded detection limits and degraded peak identification and quantification.	Stationary phase bleed itself. Quantitative inert probe in the standard.	Measure the bleed at highest method temperature relative to that at starting temperature. Compare the signal level of background at a given high temperature to that of an inert reference peak. Use a signal/noise for low concentration critical probe(s).
Response changes	Response usually drifts down leading to under-reporting or false negatives, but can go up, exaggerating results as well. MS response drift due to a dirty source can change ion ratios, affecting target compound confirmation as well as quantification.	Use a quantitative standard or multiple standards with analyte concentrations spanning the calibration range (cover at least highest and low levels).	Even though the common practice is to adjust response factors based on results, a maintenance decision is usually based on the absolute change in response or response difference relative to a reference. Signal/noise is sometimes used instead of signal alone.
Leaks	Leaks can come from anywhere: in the inlet septum, inlet top and bottom weldments, any column connections, gas supply lines. Flow related leaks generally affect areas and area repeatabilities. Pressure related leaks generally affect retention times.	Stable compounds (not affected by activity or degradation issues) like saturated hydrocarbons in quantitative amounts. Air background in air sensitive detectors.	Leaks in gas lines can be seen if you have an air sensitive detector (like mass spec, HID, or TCD) but are difficult to detect otherwise. Compare absolute response and repeatability to reference. Compare retention times and repeatability to reference.

APPLICATIONS

- Quantification of pollutants in environment and food
- Quantification of pesticide residues in food, soil and water
- Analysis of unknown hazardous organic compounds in waste dumps
- Quantification of drugs and metabolites in human blood etc
- Analysis of industrial products for quality control
- Analyze the volatiles from plant samples
- Petroleum products analysis

REFERENCES

- *FoujanFalaki. 2018.*Sample Preparation Techniques for Gas Chromatography. DOI: 10.5772/intechopen.84259
- https://blog.sepscience.com/gaschromatography/preventative-gc-maintenance-and-performance-verification-testing

LECTURE 15

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: PRINCIPLE, INSTRUMENTATION AND OPERATION

High performance liquid chromatography (HPLC) or high-speed liquid chromatography (HSLC) or high-pressure liquid chromatography (HPLC) or high sensitivity liquid chromatography (HSLC) is the synonyms used for Liquid chromatography. The generally accepted one is high performance liquid chromatography (HPLC).

PRINCIPLE

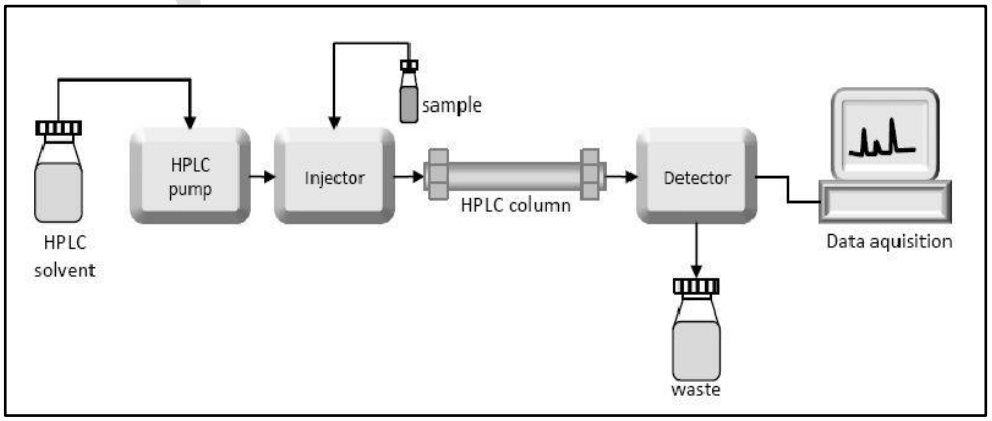
The process involves the interaction of the analyte in the sample (which travels along with a mobile phase) across an immobile surface (stationary phase) when it is forced through the column by high pressure delivered by a pump. The compounds bind at specific regions of stationary phase based on certain physical and chemical properties (Polarity, Charge, Molecular weight, Present of functional group). These bound molecules are then eluted with a suitable solvent /buffer and the same are collected with time.

INSTRUMENTATION

HPLC is an advanced technique of column liquid chromatography. It is a popular analytical technique used for the separation, identification and quantification of each constituent of mixture. It yields high performance and high speed compared with traditional column chromatography because of the forcibly pumped mobile phase. The major components of HPLC are solvent reservoir, pump, injector, column, detector and integrator.

1. Solvent Reservoir:

In HPLC the mobile phase or solvent is a mixture of polar and non-polar liquid components and its use depends on the composition of sample and polarity of the molecule to be determined.



2. Pump:

The most important and expensive part of the HPLC is the pump. Fluid flow essential for any HPLC separation is accomplished with a high-pressure pump. The pump sucks the mobile phase from solvent reservoir and forces it to column and then passes to detector. Slower the solvent flow rate, better the separation. A typical flow rate for analytical separations is 0.5 to 2.0 ml/min. The pump pressure is normally between 400-600 bar. Based on the number pumps in HPLC, it is called as isocratic (one), binary (two) and quaternary (four) model HPLC. In isocratic separations, the eluent composition remains unchanged during the analysis. Other pumps are used to perform a gradient analysis which allows composition of eluent to be changed during the analysis.

3. Injector

A sample is injected into the flow stream of the mobile phase. This is accomplished via a manual injector or an auto sampler. Typical sample volumes are 5-to 20-microliters. Commonly used type is rheodyne injector (loop injector) fixed near the pump.

4. Column and separation mode

Columns are the heart of chromatographic instruments where the compound separation takes place. It is stationary phase and provides separation through high pressure created by the small particles. Normally C_{18} and C_8 columns are used for agrochemical and environmental sample analysis.

Separation of a sample in to its constituent parts occurs because of the differences in relative affinities of different molecules for the mobile phase and stationary phase. Components that have a higher affinity for the mobile phase compared with the stationary phase migrate more rapidly, while components that have a higher affinity for the stationary phase are eluted from the column later. The order and resolution of the components emerging from column depend on the type of selected stationary and mobile phases. The time at which a specific analyte elutes (comes out of the end of the column) is called ***retention time***. The separation methods are classified into four modes viz., **adsorption, partition, ion exchange,** and **size exclusion.**Lengthy columns are preferred for separation of many compounds in mixture while short columns are used to separate simple mixtures.

Partition modes and HPLC types

*Normal phase HPLC:*This method separates analytes based on polarity. NP-HPLC uses a polar stationary phase mostly silica and a non-polar mobile phase such as hexane, chloroform and diethyl ether. The polar analyte interacted with and is retained by the polar stationary phase for longer duration.

*Reversed phase HPLC:*It has a non-polar stationary phase and an aqueous, moderately polar mobile phase. It operates on the principle of hydrophobic interactions, which result from repulsive forces

between a polar eluent, the relatively non-polar analyte, and the non-polar stationary phase. Hence more non-polar material is retained for long time. Suitable solvents are acetonitrile and methanol.

5. Detector:

The separated components from column are detected by a detector by providing electrical output signals proportional to column effluent concentration. It quantifies and identifies the sample components and provides information to the computer. Most common detector is UV-VIS detector followed by electrochemical detector, refractive index and fluorescence detectors. Mass spectrometer can also be hyphenated. The spectrophotometric detectorincludes UV, UV-VIS and photodiode array (PDA) or diode array (DAD) detectors. The electrochemical detectors have a range to detect at a minimum of picograms while remaining is efficient at nanograms and above.

6. Recorder / Computer:

Signal from the detector are collected electronically for process, store and reprocess chromatographic data. The computer integrates the response of the detector to each component and places it into a chromatograph that is easy to read and interpret.

OPEARTION

The sample mixture to be separated and analyzed is introduced, in a discrete small volume (typically microliters), into the stream of mobile phase percolating through the column. The components of the sample move through the column at different velocities, which are a function of specific physical interactions with the adsorbent (also called stationary phase). The velocity of each component depends on its chemical nature, nature of stationary phase (column) and the composition of the mobile phase. The time at which a specific analyte elutes (emerges from the column) is called its retention time. The retention time measured under particular conditions is an identifying characteristic of a given analyte.

The choice of mobile phase components, additives (such as salts or acids) and gradient conditions depends on the nature of the column and sample components. The choice of detector depends on the analyte properties.

TYPES OF HPLC BASED ON SEPARATION METHODS

i. ***Normal Phase Chromatography:*** Separation of polar analytes by partitioning into a polar, bonded stationary phase.

ii. ***Reversed Phase Chromatography:*** Separation of non-polar analytes by partitioning into a non-polar, bonded stationary phase.

iii. ***Adsorption Chromatography:*** In Between Normal and Reversed. Separation of moderately polar analytes using adsorption into a pure stationary phase (e.g. alumina or silica)

iv. ***Ion Chromatography:*** Separation of organic and inorganic ions by their partitioning into ionic stationary phases bonded to a solid support.

v. ***Size Exclusion Chromatography:*** Separation of large molecules based on the paths they take through a "maze" of tunnels in the stationary phase.

GAS CHROMATOGRAPHY VS HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Unlike gas chromatography, the liquid chromatography can safely used to separate a very wide range of organic compounds, from small-molecule drug metabolites to peptides and proteins and both volatile and nonvolatile and thermally fragile molecules.

Relative advantages and disadvantages of GC versus HPLC

S.no.	GC	HPLC
1.	Sample must be volatile or derivatized previous to GC analysis	Both volatile and non volatile compounds can be analysed; Solubility in the mobile phase is critical for analysis.
2.	Most analytes have a molecular weight (MW) below 500 Da (due to volatility issues)	No upper molecular weight limit as far as the sample can be dissolved in the appropriate mobile phase
3.	Can be coupled to MS. Several mass spectral libraries are available if using electron ionization	Methods must be adapted before using an MS detector (non-volatile buffers cannot be used)
4.	Can be coupled to several detectors depending on the application	For some detectors the solvent must be an issue. When changing detectors some methods will require prior modification

REFERENCES

- https://en.wikipedia.org/wiki/High-performance_liquid_chromatography
- https://www.slideshare.net/Alakesh0007/hplc-principleinstrumentation-and-application?from_action=save
- https://www.waters.com/waters/en_IN/HPLC-Separation-modes/nav.htm?cid=10049076&locale=en_IN
- Janaki,P., C. Bharathi and C. Chinnusamy. 2018. Techniques for herbicide residue analysis in soil, water and crops. 2018. In: Advanced Weed Management Technology options for Crop Production in Indian Agriculture. Published by Thannambikkai Publication, Coimbatore - 641 041. ISBN: 978-93-87314-57-3.

LECTURE 16
HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: SAMPLE PREPARATION, METHOD DEVELOPMENT, MAINTENANCE AND TROUBLESHOOTING

Sample preparation

It is the manipulation of samples to prior to the analysis

Why performing sample preparation for HPLC?

- Compatibility to further analysis
- Simplify complex samples
- Remove interferences from the matrix
- Concentrate or dilute the sample
- Speed of analysis
- System Robustness

METHODS OF SAMPLE PREPARATION

It varies and depends on the nature of sample viz., liquid, solid or gas. Volatile analytes that are labile, thermally unstable, or prone to adsorb to metal surfaces in the vapour state are analysed well by HPLC.

Gaseous sample: Trapping is required to analyze gaseous samples by HPLC. The gas sample is either (a) passed through a solid support and subsequently eluted with a solubilising liquid, or (b) bubbled through a liquid that traps the analyte(s).

Solid samples: Samples that are solid (or semi-solid) must usually be put into a liquid form. The analytes must be extracted from the insoluble solid matrix using suitable solvents by shaking, centrifugation or vortexing etc. If the solvent-extractable portion of a solid sample is of interest, then techniques such as liquid-solid extraction, supercritical fluid extraction, microwave-assisted extraction, Soxhlet extraction, or pressurized fluid extraction can be used. Here, the solid material is exposed to a solubilising liquid or supercritical fluid (usually carbon dioxide, often doped with a polar solvent such as methanol), sometimes with added heat and/or pressure. Sample components soluble in the liquid eventually are totally or partially leached out of the sample. Obviously, the more porous the sample and the more finely divided the solid sample, the easier it is to extract components.

If the entire solid sample is to be analyzed, more drastic dissolution techniques or stronger solvents may be required. Once analytes have been quantitatively extracted from a solid sample, the resulting liquid fraction can either be injected directly into the HPLC or GC instrument, or subjected to further pre-treatment.

SAC 454

The commonly used techniques for the extraction of analyte from agricultural samples are detailed below.

s.no.	Nature of sample	Method of Sample Pre-treatment	Principles of Technique
11.	Soil, plant and environmental samples	Solid-Liquid Extraction	Solvent is added to dissolves/extracts/leaches the analyte of interest; solution is separated from solid by filtration (sometimes called "shake/filter" method)
12.	Plant, grain and oil seeds etc.	Soxhlet Extraction	Constantly refluxing fresh solvent flows through the sample in thimble and dissolves analytes that are continuously collected in a boiling flask
13.	Fresh plant and seed samples	Homogenization	Sample is blended or a mechanically homogenized using solvent; solvent is removed for further workup.
14.	Soil and environmental samples	Sonication	Use of ultrasound to create vigorous agitation at the surface of a finely divided solid material.
		Dissolution	Sample is treated with dissolving solvent and taken directly into solution with or without chemical change.
		Solid Phase Extraction (**SPE**)	Sample is applied to, and liquid is passed through, a column packed solid phase that selectively removes analyte or interferences; analyte can be eluted with strong solvent.
		Liquid-Liquid Extraction	Sample is partitioned between two immiscible phases which are chosen to maximize differences in solubility; interference-free

SAC 454

			analytes are then recovered from one of the two phases.
		Dilution	Sample is diluted with compatible solvent with HPLC mobile phase
		Evaporation	Liquid is removed by gentle heating at atmospheric pressure with flowing air or inert gas; vacuum is useful for low volatility liquids.
		Distillation	Sample is heated to boiling point of solvent and volatile analytes in the vapor phase are condensed and collected.
		Micro dialysis	A semi-permeable membrane is placed between two aqueous liquid phases and analytes transfer from one liquid to the other, based on differential concentration.
15.	Liquids & Suspensions	Filtration	Liquid is passed through paper or membrane filter or SPE cartridge/disk to remove suspended particulates.
16.	Liquids & Suspensions	Centrifugation	Sample is placed in tapered centrifuge tube and spun at high force (thousands to hundreds of thousands times gravity); supernatant liquid is decanted.
17.	Liquids & Suspensions	Sedimentation	Sample is allowed to settle when left undisturbed in a sedimentation tank; settling rate dependent on Stoke's radius.

Either single or combinations of the above methods are used to extract the analyte from sample matrix. Solid phase extraction is one of the more widely used sample preparation techniques for liquid samples or solid samples that have been put into a liquid form by dissolution or extraction. SPE can also

be used for certain gaseous sample by trapping them on a sorbent or by in situ derivatization using reactive chemicals.

QuEChERS (Quick Easy Cheap Effective Rugged Safe) method

QuEChERS have recently gained a great deal of attention, especially for the extraction of pesticides from fruit and vegetable samples. In 2003, Anastassiades et al. reported an acetonitrile based method for sample preparation called as QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe). The QuEChERS technique combines liquid-liquid extraction with dispersive solid phase extraction.

What is QuEChERS?

QuEChERS is a sample preparation approach entailing solvent extraction of high-moisture samples with acetonitrile, ethyl acetate, or acetone and partitioning with magnesium sulphate alone or in combination with other salts followed by clean up using d-SPE. It is very flexible and since its inception, there have been several modifications of the technique can be made depending on analytes, matrices, instrumentation and analyst preferences.

This method covers a very wide scope of analytes, including polar, semi-polar and non-polar pesticide residues in various food matrices.

The procedure involves:

- initial single-phase extraction of the sample with acetonitrile in the presence of high amounts of salts (for example, sodium chloride and magnesium sulphate) and buffering agents (for example, citrate).
- Removal of water and clean-up are performed simultaneously in an aliquot of the acetonitrile extract with dispersive solid phase extraction using $MgSO_4$and primary secondary amine (PSA) sorbent (removes protein and organic acids).

METHOD DEVELOPMENT

Method development encompasses many stages and can take months to complete, depending on the complexity and goals of the method. The important aspects to be considered while doing method development are discussed below.

(i). Sample type

- Free from interferences, should not damage column and compatible with HPLC

(ii). Type of chromatography

- Reverse phase/ normal phase/ion exchange/size exclusion etc

(***iii). Column selection***

- Knowledge of sample, interaction of components with packing material and properties of packing material.

(iv). Detector setting

- Select suitable detector from the wide range deepening on sample and analyte nature. Few are given below.

Detector	**Sample suitability**
UV detector	• Detects only substances which absorb light in UV wavelength range • Detects all samples which contain chromophores
Fluorescence detector	• Detects eluted solutes on basis of fluorescence • For trace analysis
Electrical conductivity detector	• Used with ion suppressor column • •To allow salts and buffers to be used in mobile phase without affecting detector output
Refractive Index Detector	• Least sensitive • Only when other detectors are inappropriate • Can handle concentration without overloading the detector

(v). Selection of mobile phase composition

- Organic phase concentration required for mobile phase can be estimated by gradient elution method.
- Elution strength of mobile phase depends upon its polarity.
- Ionic samples can be separated if they are present in un-dissociated form.
- If retention time is too long, increase in organic phase concentration is required.
- If tailing or fronting occurs, the mobile phase is not totally compatible with the solutes.

(vi). Develop the method

a). stepwise incremental (one-factor-at-a-time) approach based on results from previous experiment

b). systematic screening protocol, in which you evaluate factors such as stationary phases, solvents, and pH, and column chemistry to fine-tune selectivity and retention and thereby enhance resolution.

(vii). Select a standardization technique

If required, such as an internal or external standard. For example, a PDA detector can be used to investigate the linearity of the active pesticide ingredient and related substances in the proposed concentration range.

SAC 454

(viii). Check overall performance of the analysis technique

- Performance requirements can include variables such as accuracy, precision, reproducibility, linearity, limits of detection and limits of quantitation.

(ix).Verify method optimization and robustness.

- Use an experimental design approach to determine the experimental factors that have significant impact on the method: ie
  a) HPLC conditions like: % organic, pH, flow rate, temperature, wavelength, column age.
  b) Sample preparation: e.g., % organic, pH, shaking/sonication, sample size, sample age.
  c) Calculation/standardization: e.g., integration, wavelength, standard concentration, response factor correction.

(x). Validating method.

- The goal is to demonstrate that results from the method performance will not be significantly impacted by slight variations of the method conditions.

MAINTENACE AND TROUBLE SHOOTING

Some troubles may happen when the actual sample is analyzed though the troubles never happen when standards are analyzed. In most cases, they are caused by inappropriate selection of the solvent in which the sample is dissolved. The details of troubles frequently occurred during HPLC operation and possible rectification and maintenance tips are given in the table below.

Troubles	Reasons	Rectification	Prevention Techniques
High pressure	• High flow rate • Plugged frit • Column contamination Plugged packing	Check pressure with/without column - many pressure problems are due to blockages in the system or guard col. If pressure reduced, then problem is due to column and can be solved by. a) *Back flush column* – Clear "dirty" frit surface *b) Wash column* • Eliminate column contamination and	• Use column protection like in-line filters& guard columns • Filter samples • Filter buffered mobile phases • Sample clean-up (i.e. SPE) • Appropriate column flushing

		plugged packing • high molecular weight/adsorbed compounds precipitate from sample or buffer cleared • Change frit – Clear plugged frit	
Low Pressure	• Leak • Incorrect flow • Improper column • Column temperature too high	• Disconnect each component and locate the leakage point • Change flow rate or composition and observe the pressure	• Adjust flow rate • Locate and correct • Use proper column • Lower temperature
Split peaks	• Disrupted sample path by partially plugged frit column contamination • Sample solvent incompatible with mobile phase	• Cleaning frit or replacing • Column washing eliminates the peak splitting • Change solvent	• Periodical washing of frit and column • Whenever possible, inject samples in mobile phase.
Peak tailing/fronting Broad peaks Loss of Efficiency	• Column "secondary interactions"	• Column contamination • Column aging • Column loading • Extra-column effects	• Avoiding secondary interaction by using mobile phase modifier (TEA) • Keeping organic concentration in sample solvent < mobile phase • Evaluate both volume and mass loading and dilute if high before injection
Unknown "Phantom" Peaks/ ghost	• Impurities in the sample, reagents or material	• Inject small sample volumes. • Use a low-volume	• Changing clean-up procedure and/or check possible source of

peaks	• very late eluting peak from a preceding run	detector cell.	contamination • Increasing run time or gradient slope and/or increase flow rate
Instability of retention time.	• Column aging • Column contamination • Insufficient equilibration • Poor column/mobile phase combination • Change in mobile phase • Change in flow rate • Different gradient delay volumes	• Checking all causes of column-to-column change • Checking the method ruggedness (buffers/ionic strength) as *buffers are critical to good retention and peak shape in many separations.*	• Using columns designed for chosen pH • pH sensitivity (sample/column interactions) • Avoiding the use of mobile phase very close to an analytepKa
Noisy baseline	• Dirty flow cell • Detector lamp failing • Pulses from pump if periodically • Temperature effects on detector • Air bubbles passed through detector	• Degas mobile phase. • Flush system to remove air from detector cell or pump. • Check system for loose fittings. • Check pump for leaks, salt build-up, and unusual noise. • Change pump seals if necessary. • Mix mobile phase or use less viscous sample. • Reduce differential or add heat exchanger	
Baseline drift	• Gradient elution • temperature unstable • Contamination in mobile phase • Mobile phase not in equilibrium with column	• Control column and mobile phase temperature, use heat exchanger before detector • Use HPLC grade solvents, high purity salts, and additives. • Degas mobile phase before use	

SAC 454

References

1. https://www.shodex.com/en/da1/02/08.html#!
2. https://www.waters.com/webassets/cms/support/docs/715003355_dev_meth.pdf
3. https://shodhganga.inflibnet.ac.in/bitstream/10603/22707/8/08_chapter%203.pdf
4. Anastassiades et al., J. AOAC Int., 86, 412–431(2003).
5. TADEO ET AL.2012. Review of Sample Preparation Techniques for the Analysis of Pesticide Residues in Soil. Journal Of AOAC International VOL. 95, No. 5,. pp. 1258-1271.
6. www.quechers.com/ (follow link to "QuEChERS — A Mini-Multiresidue Method for the Analysis of Pesticide Residues in Low-Fat Products").
7. https://www.sciencedirect.com/science/article/abs/pii/S221415881830028X

SAC 454

LECTURE 16

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: SAMPLE PREPARATION, METHOD DEVELOPMENT, MAINTENANCE AND TROUBLESHOOTING

Sample preparation

It is the manipulation of samples to prior to the analysis

Why performing sample preparation for HPLC?

- Compatibility to further analysis
- Simplify complex samples
- Remove interferences from the matrix
- Concentrate or dilute the sample
- Speed of analysis
- System Robustness

METHODS OF SAMPLE PREPARATION

It varies and depends on the nature of sample viz., liquid, solid or gas. Volatile analytes that are labile, thermally unstable, or prone to adsorb to metal surfaces in the vapour state are analysed well by HPLC.

Gaseous sample: Trapping is required to analyze gaseous samples by HPLC. The gas sample is either (a) passed through a solid support and subsequently eluted with a solubilising liquid, or (b) bubbled through a liquid that traps the analyte(s).

Solid samples: Samples that are solid (or semi-solid) must usually be put into a liquid form. The analytes must be extracted from the insoluble solid matrix using suitable solvents by shaking, centrifugation or vortexing etc. If the solvent-extractable portion of a solid sample is of interest, then techniques such as liquid-solid extraction, supercritical fluid extraction, microwave-assisted extraction, Soxhlet extraction, or pressurized fluid extraction can be used. Here, the solid material is exposed to a solubilising liquid or supercritical fluid (usually carbon dioxide, often doped with a polar solvent such as methanol), sometimes with added heat and/or pressure. Sample components soluble in the liquid eventually are totally or partially leached out of the sample. Obviously, the more porous the sample and the more finely divided the solid sample, the easier it is to extract components.

If the entire solid sample is to be analyzed, more drastic dissolution techniques or stronger solvents may be required. Once analytes have been quantitatively extracted from a solid sample, the resulting liquid fraction can either be injected directly into the HPLC or GC instrument, or subjected to further pre-treatment.

The commonly used techniques for the extraction of analyte from agricultural samples are detailed below.

s.no.	Nature of sample	Method of Sample Pre-treatment	Principles of Technique
18.	Soil, plant and environmental samples	Solid-Liquid Extraction	Solvent is added to dissolves/extracts/leaches the analyte of interest; solution is separated from solid by filtration (sometimes called "shake/filter" method)
19.	Plant, grain and oil seeds etc.	Soxhlet Extraction	Constantly refluxing fresh solvent flows through the sample in thimble and dissolves analytes that are continuously collected in a boiling flask
20.	Fresh plant and seed samples	Homogenization	Sample is blended or a mechanically homogenized using solvent; solvent is removed for further workup.
21.	Soil and environmental samples	Sonication	Use of ultrasound to create vigorous agitation at the surface of a finely divided solid material.
		Dissolution	Sample is treated with dissolving solvent and taken directly into solution with or without chemical change.
		Solid Phase Extraction (**SPE**)	Sample is applied to, and liquid is passed through, a column packed solid phase that selectively removes analyte or interferences; analyte can be eluted with strong solvent.
		Liquid-Liquid Extraction	Sample is partitioned between two immiscible phases which are chosen to maximize differences in solubility; interference-free

SAC 454

			analytes are then recovered from one of the two phases.
		Dilution	Sample is diluted with compatible solvent with HPLC mobile phase
		Evaporation	Liquid is removed by gentle heating at atmospheric pressure with flowing air or inert gas; vacuum is useful for low volatility liquids.
		Distillation	Sample is heated to boiling point of solvent and volatile analytes in the vapor phase are condensed and collected.
		Micro dialysis	A semi-permeable membrane is placed between two aqueous liquid phases and analytes transfer from one liquid to the other, based on differential concentration.
22.	Liquids & Suspensions	Filtration	Liquid is passed through paper or membrane filter or SPE cartridge/disk to remove suspended particulates.
23.	Liquids & Suspensions	Centrifugation	Sample is placed in tapered centrifuge tube and spun at high force (thousands to hundreds of thousands times gravity); supernatant liquid is decanted.
24.	Liquids & Suspensions	Sedimentation	Sample is allowed to settle when left undisturbed in a sedimentation tank; settling rate dependent on Stoke's radius.

Either single or combinations of the above methods are used to extract the analyte from sample matrix. Solid phase extraction is one of the more widely used sample preparation techniques for liquid samples or solid samples that have been put into a liquid form by dissolution or extraction. SPE can also

be used for certain gaseous sample by trapping them on a sorbent or by in situ derivatization using reactive chemicals.

QuEChERS (Quick Easy Cheap Effective Rugged Safe) method

QuEChERS have recently gained a great deal of attention, especially for the extraction of pesticides from fruit and vegetable samples. In 2003, Anastassiades et al. reported an acetonitrile based method for sample preparation called as QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe). The QuEChERS technique combines liquid-liquid extraction with dispersive solid phase extraction.

What is QuEChERS?

QuEChERS is a sample preparation approach entailing solvent extraction of high-moisture samples with acetonitrile, ethyl acetate, or acetone and partitioning with magnesium sulphate alone or in combination with other salts followed by clean up using d-SPE. It is very flexible and since its inception, there have been several modifications of the technique can be made depending on analytes, matrices, instrumentation and analyst preferences.

This method covers a very wide scope of analytes, including polar, semi-polar and non-polar pesticide residues in various food matrices.

The procedure involves:

- initial single-phase extraction of the sample with acetonitrile in the presence of high amounts of salts (for example, sodium chloride and magnesium sulphate) and buffering agents (for example, citrate).
- Removal of water and clean-up are performed simultaneously in an aliquot of the acetonitrile extract with dispersive solid phase extraction using $MgSO_4$and primary secondary amine (PSA) sorbent (removes protein and organic acids).

METHOD DEVELOPMENT

Method development encompasses many stages and can take months to complete, depending on the complexity and goals of the method. The important aspects to be considered while doing method development are discussed below.

(i). Sample type

- Free from interferences, should not damage column and compatible with HPLC

(ii). Type of chromatography

- Reverse phase/ normal phase/ion exchange/size exclusion etc

(***iii). Column selection***

- Knowledge of sample, interaction of components with packing material and properties of packing material.

(iv). Detector setting

- Select suitable detector from the wide range deepening on sample and analyte nature. Few are given below.

Detector	**Sample suitability**
UV detector	• Detects only substances which absorb light in UV wavelength range • Detects all samples which contain chromophores
Fluorescence detector	• Detects eluted solutes on basis of fluorescence • For trace analysis
Electrical conductivity detector	• Used with ion suppressor column • •To allow salts and buffers to be used in mobile phase without affecting detector output
Refractive Index Detector	• Least sensitive • Only when other detectors are inappropriate • Can handle concentration without overloading the detector

(v). Selection of mobile phase composition

- Organic phase concentration required for mobile phase can be estimated by gradient elution method.
- Elution strength of mobile phase depends upon its polarity.
- Ionic samples can be separated if they are present in un-dissociated form.
- If retention time is too long, increase in organic phase concentration is required.
- If tailing or fronting occurs, the mobile phase is not totally compatible with the solutes.

(vi). Develop the method

a). stepwise incremental (one-factor-at-a-time) approach based on results from previous experiment

b). systematic screening protocol, in which you evaluate factors such as stationary phases, solvents, and pH, and column chemistry to fine-tune selectivity and retention and thereby enhance resolution.

(vii). Select a standardization technique

If required, such as an internal or external standard. For example, a PDA detector can be used to investigate the linearity of the active pesticide ingredient and related substances in the proposed concentration range.

SAC 454

(viii). Check overall performance of the analysis technique

- Performance requirements can include variables such as accuracy, precision, reproducibility, linearity, limits of detection and limits of quantitation.

(ix).Verify method optimization and robustness.

- Use an experimental design approach to determine the experimental factors that have significant impact on the method: ie
  d) HPLC conditions like: % organic, pH, flow rate, temperature, wavelength, column age.
  e) Sample preparation: e.g., % organic, pH, shaking/sonication, sample size, sample age.
  f) Calculation/standardization: e.g., integration, wavelength, standard concentration, response factor correction.

(x). Validating method.

- The goal is to demonstrate that results from the method performance will not be significantly impacted by slight variations of the method conditions.

MAINTENACE AND TROUBLE SHOOTING

Some troubles may happen when the actual sample is analyzed though the troubles never happen when standards are analyzed. In most cases, they are caused by inappropriate selection of the solvent in which the sample is dissolved. The details of troubles frequently occurred during HPLC operation and possible rectification and maintenance tips are given in the table below.

Troubles	Reasons	Rectification	Prevention Techniques
High pressure	• High flow rate • Plugged frit • Column contamination Plugged packing	Check pressure with/without column - many pressure problems are due to blockages in the system or guard col. If pressure reduced, then problem is due to column and can be solved by. c) *Back flush column* – Clear "dirty" frit surface *d) Wash column* • Eliminate column contamination and	• Use column protection like in-line filters& guard columns • Filter samples • Filter buffered mobile phases • Sample clean-up (i.e. SPE) • Appropriate column flushing

		plugged packing • high molecular weight/adsorbed compounds precipitate from sample or buffer cleared • Change frit – Clear plugged frit	
Low Pressure	• Leak • Incorrect flow • Improper column • Column temperature too high	• Disconnect each component and locate the leakage point • Change flow rate or composition and observe the pressure	• Adjust flow rate • Locate and correct • Use proper column • Lower temperature
Split peaks	• Disrupted sample path by partially plugged frit column contamination • Sample solvent incompatible with mobile phase	• Cleaning frit or replacing • Column washing eliminates the peak splitting • Change solvent	• Periodical washing of frit and column • Whenever possible, inject samples in mobile phase.
Peak tailing/fronting Broad peaks Loss of Efficiency	• Column "secondary interactions"	• Column contamination • Column aging • Column loading • Extra-column effects	• Avoiding secondary interaction by using mobile phase modifier (TEA) • Keeping organic concentration in sample solvent < mobile phase • Evaluate both volume and mass loading and dilute if high before injection
Unknown "Phantom" Peaks/ ghost	• Impurities in the sample, reagents or material	• Inject small sample volumes. • Use a low-volume	• Changing clean-up procedure and/or check possible source of

SAC 454

peaks	• very late eluting peak from a preceding run	detector cell.	contamination • Increasing run time or gradient slope and/or increase flow rate
Instability of retention time.	• Column aging • Column contamination • Insufficient equilibration • Poor column/mobile phase combination • Change in mobile phase • Change in flow rate • Different gradient delay volumes	• Checking all causes of column-to-column change • Checking the method ruggedness (buffers/ionic strength) as *buffers are critical to good retention and peak shape in many separations.*	• Using columns designed for chosen pH • pH sensitivity (sample/column interactions) • Avoiding the use of mobile phase very close to an analytepKa
Noisy baseline	• Dirty flow cell • Detector lamp failing • Pulses from pump if periodically • Temperature effects on detector • Air bubbles passed through detector	• Degas mobile phase. • Flush system to remove air from detector cell or pump. • Check system for loose fittings. • Check pump for leaks, salt build-up, and unusual noise. • Change pump seals if necessary. • Mix mobile phase or use less viscous sample. • Reduce differential or add heat exchanger	
Baseline drift	• Gradient elution • temperature unstable • Contamination in mobile phase • Mobile phase not in equilibrium with column	• Control column and mobile phase temperature, use heat exchanger before detector • Use HPLC grade solvents, high purity salts, and additives. • Degas mobile phase before use	

SAC 454

References

8. https://www.shodex.com/en/da1/02/08.html#!
9. https://www.waters.com/webassets/cms/support/docs/715003355_dev_meth.pdf
10. https://shodhganga.inflibnet.ac.in/bitstream/10603/22707/8/08_chapter%203.pdf
11. Anastassiades et al., J. AOAC Int., 86, 412–431(2003).
12. TADEO ET AL.2012. Review of Sample Preparation Techniques for the Analysis of Pesticide Residues in Soil. Journal Of AOAC International VOL. 95, No. 5,. pp. 1258-1271.
13. www.quechers.com/ (follow link to "QuEChERS — A Mini-Multiresidue Method for the Analysis of Pesticide Residues in Low-Fat Products").
14. https://www.sciencedirect.com/science/article/abs/pii/S221415881830028X

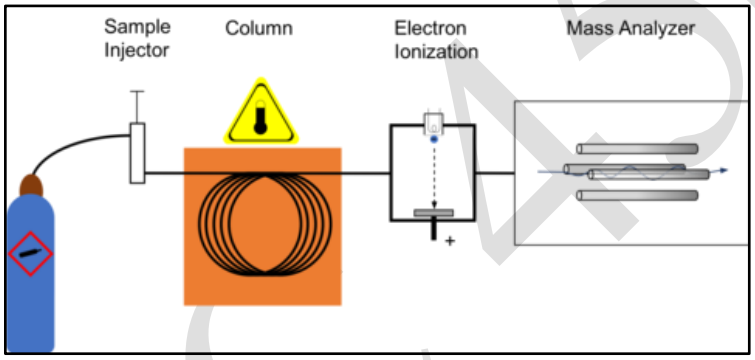
SAC 454

LECTURE 17

GC – MS: PRINCIPLE, INSTRUMENTATION, SAMPLE PREPARATION, METHOD DEVELOPMENT, MAINTENANCE AND TROUBLESHOOTING

GAS CHROMATOGRAPHY-MASS SPECTROSCOPY

Gas chromatography-mass spectroscopy (GC-MS) is one of the so-called hyphenated analytical techniques. As the name implies, it is actually ***two techniques that are combined to form a single method of analyzing mixtures of chemicals***. Gas chromatography separates the components of a mixture and mass spectroscopy characterizes each of the components individually. By combining the two techniques, an analytical chemist can both qualitatively and quantitatively evaluate a solution containing a number of chemicals.



Components of GC-MS

The principle, components and instrumentation of the GC was already you studied in previous lectures. Hence the MS is discussed here.

MASS SPECTROMETRY (MS)

- It is an analytical technique that ionizes chemical species and sorts the ions based on their mass-to-charge ratio. In simpler terms, a mass spectrum measures the masses within a sample.
- Mass spectrometry is used in different fields and is applied to pure samples as well as complex mixtures.
- A mass spectrum is a plot of the ion signal as a function of the mass-to-charge ratio.

These spectra are used to determine the elemental or isotopic signature of a sample, the masses of particles and molecules, and to elucidate the chemical structures of molecules, such as peptides and other chemical compounds. The development of hyphenated methods combining diverse separation techniques with mass spectrometric detectors has generated an enormous application on speciation analysis in soil environmental studies.

History

The history of mass spectrometry has its heredity in physical and chemical studies concerning the nature of matter. The study of gas discharges in the mid 19th century led to the discovery of anode and cathode rays, which turned out to be positive ions and electrons. J.J. Thomson discovered the nature of cathode rays and measured their mass/electron (m/e) ratios and built an apparatus to measure the m/e values. Improved capabilities in the separation of these positive ions enabled the discovery of stable isotopes of the elements. The first such discovery was with the element neon, which was shown by mass spectrometry to have at least two stable isotopes: ^{20}Ne (neon with 10 protons and 10 neutrons) and ^{22}Ne (neon with 10 protons and 12 neutrons). Mass spectrometers were used in the Manhattan project for the uranium isotopes separation necessary to create the atomic bomb. Early applications in chemistry included bond energies, ionization energies and other thermo chemical determinations. In 1940, chemical (*viz*., molecular) analysis began with the use of mass spectrometric fragmentation patterns to characterize petroleum distillates. Now its application has been extended to study the isotopic compositions of the element, protein sequencing, mapping etc.

PRINCIPLE OF MS AND ITS COMPONENTS

Principle

A mass spectrometer is an instrument that produces ions and separates them in the gas phase according to their **mass-to-charge ratio (m/z)** and then records the relative abundance of each ion type.

When a sample is introduced into a mass spectrometer, ions are produced from the sample by electron ionisation and the molecular ion undergoes fragmentation. Fragmented ions are separated according to their mass-to-charge ratio in the mass analyzer and measured based on their abundance with the detector that converts the ions into electrical signals in the form of mass spectrum.

Ions provide information concerning the nature and the structure of their precursor molecule. In the spectrum of a pure compound, the molecular ion, if present, appears at the highest value of m/z (followed by ions containing heavier isotopes) and gives the molecular mass of the compound.

Basically, a mass spectrometric analysis can be envisioned to be made up of the following steps:

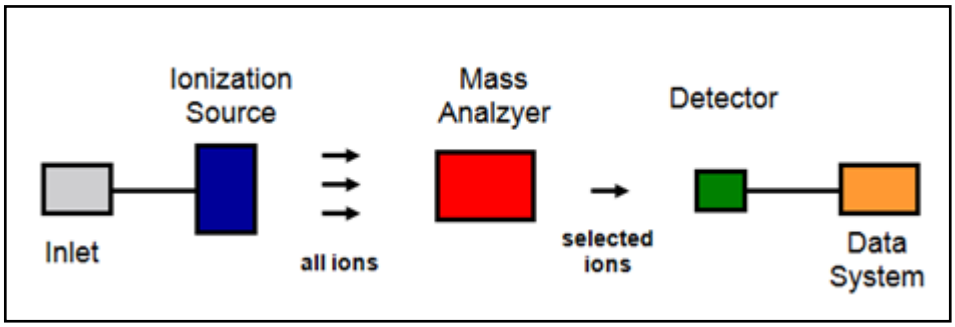
Sample Introduction→Ionization→Mass Analysis Ion Detection/Data Analysis

Components of Simple MS

The instrument consists of five major components:

1. ***Sample inlet:*** admit the samples to be studied to the ion source while maintaining the high vacuum requirements (~10-6 to 10-8 mm of mercury) of the technique.
2. ***Ion Source:*** For producing gaseous ions from the substance being studied.

3. ***Analyzer:*** For resolving the ions into their characteristics mass components according to their mass-to-charge ratio.
4. ***Detector System:*** For detecting the ions and recording the relative abundance of each of the resolved ionic species.
5. ***Recorder:***To control the instrument, acquire and manipulate data, and compare spectra to reference libraries.



Components of Mass Spectrometer

Important processes occurring in MS

a) **Ionization**

- Atoms are ionized by knocking one or more electrons off to give positive ions by bombardment with a stream of electrons. Most of the positive ions formed will carry charge of +1.
- Ionization can be achieved by the following methods in MS attached to the GC:
  - Electron Ionization (EI-MS)
  - Chemical Ionization (CI-MS)
  - Negative Chemical Ionization

b) Acceleration

- Ions are accelerated so that they all have same kinetic energy.
- Positive ions pass through 3 slits with voltage in decreasing order.
- Middle slit carries intermediate and finals at zero volts.

c) Deflection

- Ions are deflected by a magnetic field due to difference in their masses.
- The lighter the mass, more they are deflected.
- It also depends upon the no. of +ve charge an ion is carrying; the more +ve charge, more it will be deflected.

d) Detection

- The beam of ions passing through the mass analyzer is detected by detector on the basis of m/e ratio.
- When an ion hit the metal box, charge is neutralized by an electron jumping from metal on to the ion.

Types of MS analyzers used with GC:

(i). ***Quadrupole mass analysers*** - In a quadrupole mass analyser a set of four rods are arranged parallel to the direction. Here a DC current and radio frequency (RF) is applied to generate oscillating electrostatic field in between the rods. Based on this only m/z is been determined and stable oscillation takes place. And ion travels in quadrupole axis with cork screw type of trajectory.

(ii). ***Time of Flight analysers (TOF) -*** the ions entering into the chamber are trapped by electromagnetic fields and they oscillates in concentric trajectories. This process is called resonant ejection.

(iii). ***Ion trap analyser -*** the velocities of two ions are created by uniform electromagnetic force applied to all the ions at same time, causing them to accelerate down a flight tube. Lighter ions travel faster and strike the detector first so that the m/z ratio of ions is detected.

INSTRUMENTATION OF GC-MS

When two separate techniques such as gas chromatography (GC) and mass spectrometry (MS) are successfully combined to form gas chromatography mass spectrometry (GC-MS), the advantages become obvious. ***GC can separate many volatile and semi-volatile compounds but not always selectively detect them whereas MS can selectively detect many compounds but not always separate them.*** Each compound has a unique fingerprint and software is readily available to provide a library of spectra for unknown compounds. Recently, three-dimensional GC-MS has become popular.

SAMPLE PREPARATION FOR GC-MS

Normally MS is used as a hyphenated technique with GC, LC and so on. Hence the sample preparation techniques followed for GC and HPLC is hold well for MS also. If the sample is directly used, then care must be taken to have highly pure samples. A variety of strategies are used to prepare the samples for mass spectrometric analysis. The main steps during sample preparation are

- Extraction

- Partitioning of the compound by different means viz., acid hydrolysis, liquid-liquid extraction
- Derivatization depending on the analyte nature
- Clean up
- Volume reduction
- Phase change if needed
- Filtering
- Injection into GC for separation

Prepared sample should be / have

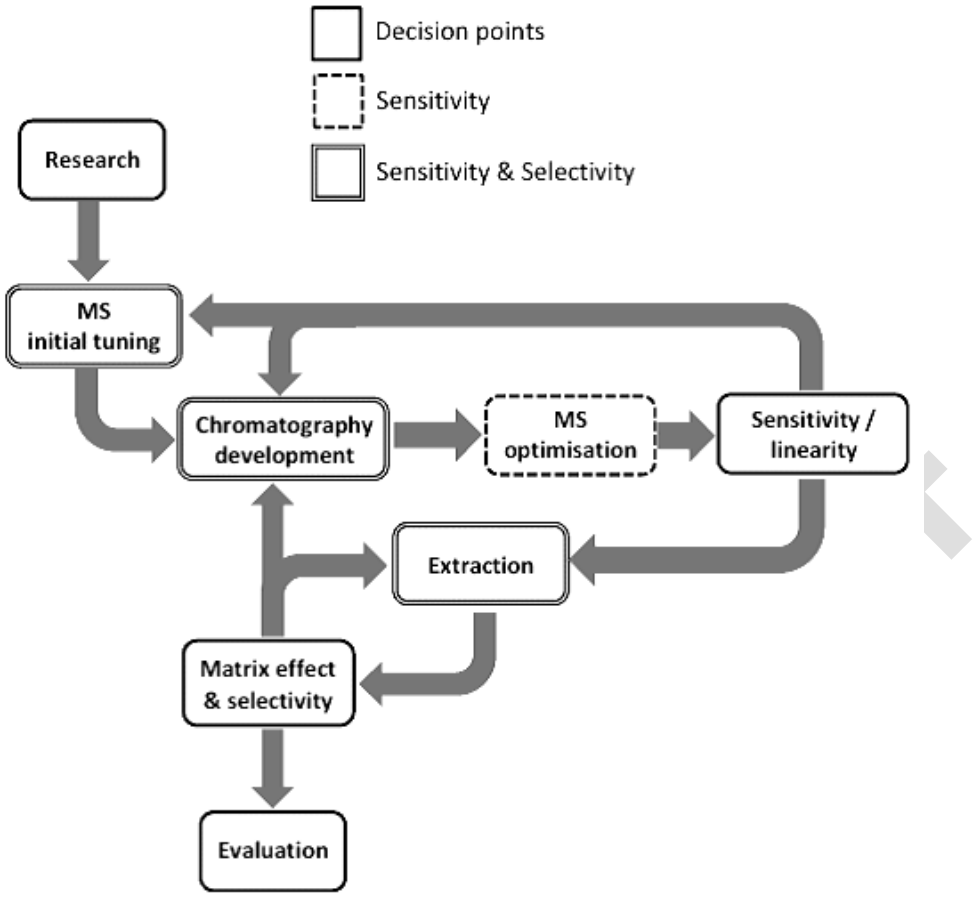
1. A sample concentration of 1 mg/ml is ideal.
2. Samples for intact protein mass analysis must be of high purity (>90%) and should be of not less than 100 microgram in quantity.
3. Salt and detergent free and should not be radioactive
4. Sample vials should be clearly labelled and sealed.
5. Solid samples can be posted in room temperature.

METHOD DEVELOPMENT

The method development involves the process of evaluating GC-MS response and developing a multiple reaction monitoring (MRM) or selective reaction monitoring (SRM) methodology that provides optimum MS condition for the detection of the compounds of interest. The method development or MS evaluation involves the assessment of

- Whether the compound can be detected by GC-MS without chemical modification
- Mode of ionization best suited for detection such as electron impact (EI) or chemical ionization (CI) etc.
- Polarity of that provides the best response

The developing a quantitative MS assay involves, iterative process that can often be very time consuming if the assay requirements are challenging. Typically the two main demands are achieving adequate ***sensitivity and selectivity***. The model flow chart of stepwise optimisation of GC-MS is given below.



MAINTENANCE AND TROUBLESHOOTING

A systematic reverse order approach to problems is always better. Keeping this in mind, the commonly seen problems, possible causes, and suggested treatment for MS are listed in the table below.

Problem	Cause	Treatment
Vacuum Systems		
• High vacuum pump would not turn on. No pressure reading	• Filament is burned out. No filament voltage. • System leak. Rough pump cannot reach starting vacuum. • Rough pump would not turn on. • Turbo pump rotor has seized.	• Shutdown vacuum. Replace filament. • Find and repair system leak. • Check rough pump power. Replace pump oil. • Replace pump.
• Cannot reach operating vacuum	• Contaminated fore or diffusion pump oil.	• Look for background increase in previous TIC. • Replace fore pump oil. Have diffusion pump oil replaced by service

		representative.
MS SOURCE AND CALIBRATION PROBLEMS		
• Narrow mass range	• scanned Widen scanned mass range	• Ions may be higher or lower in mass • Check expected mass defects
• Loss of Sensitivity	• Analyzer pressure too high • Source filament misaligned • Electron multiplier gain • Down • Ion source is dirty	• Pull source • Check multiplier gain • Clean or bake out source
• No Signal	• No ion beam	• Check isolation valve • Check filament continuity • Check for ion lens voltages • Check accelerating voltage • Check electron multiplier/detector • Check data system
• Resolution low	• Peak shape shows lift-off • Peak shape skewed • Pressure too high	• Quadrupole rods dirty • Sector slits misaligned
DATA ANALYSIS		
• Calibration error • file	• Incorrect calibration	• Check current calibration file
• High background ion • intensity	• Column bleed / loss of theoretical plates	• Check masses for fit

APPLICATIONS OF MASS SPECTROMETRY IN AGRICULTURE

1. Biomolecule characterization
   - Metabolites profiling
   - Liphophilic profiling
   - Phyto hormones and secondary metabolites
   - Dosimetric analysis
2. Environmental analysis
   - Pesticides on foods
   - Soil and groundwater contamination

SUMMARY

GC-MS is an ideal technique for qualitative and quantitative determination of ***volatile and semi-volatile organic compounds in a wide variety of samples***. A detection limit as ***low as sub-ng is possible***. The sample must be in solution for injection into the GC. This can simply be dissolution in a solvent, typically dichloromethane or extraction.

REFERENCES

- http://www.premierbiosoft.com/tech_notes/mass-spectrometry.html
- https://www.britannica.com/science/mass-spectrometry
- https://microbenotes.com/mass-spectrometry-ms-principle-working-instrumentation-steps-applications/
- https://info.gbiosciences.com/blog/sample-preparation-for-mass-spectrometric-analysis
- https://plantmethods.biomedcentral.com/articles/10.1186/s13007-018-0341-4
- http://www.ispybio.com/search/protocols/mass%20protocol3.pdf
- www.rsc.org/images/AMC%20LCMS%20Guide_tcm18-240030.pdf
- https://www.waters.com/webassets/cms/library/docs/720002710en.pdf
- https://www.rsc.org/images/AMC%20LCMS%20Guide_tcm18-240030.pdf
- http://ww2.chemistry.gatech.edu/~bostwick/stms/spec.html
- https://onlinelibrary.wiley.com/doi/pdf/10.1002/9780470228357.app2
- Sneddon, J., S. Masuram& J. C. Richert (2007): Gas Chromatography-Mass Spectrometry-Basic Principles, Instrumentation and Selected Applications for Detection of Organic Compounds, Analytical Letters, 40:6, 1003-1012. http://dx.doi.org/10.1080/00032710701300648.

SAC 454