Muhammad Sajid Hamid Akash
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Essentials of Pharmaceutical Analysis

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Dr. Kanwal Rehman is an Assistant Professor at the Department of Pharmacy, University of Agriculture Faisalabad, Pakistan. Dr. Rehman completed her PhD in Pharmacology and Toxicology at Zhejiang University, Hangzhou, China, in the course of which she did excellent work on exploring arsenic-induced carcinogenic and anticancer effects. She has a keen interest in the pathogenesis of metabolic disorders like diabetes mellitus and obesity, as well as new therapeutic modalities for their treatment. She has published more than 90 articles in peer-reviewed journals, as well as 26 book chapters with international publishers like Springer Nature, Elsevier, Material Research Forum LLC, and CRC Press. She is currently working on three research projects funded by the Higher Education Commission (HEC) of Pakistan and previously completed one HEC-funded project. Currently, she is investigating treatment strategies like the use of natural biogenic compounds against different risk factors for metabolic disorders including pancreatitis, cardiometabolic disorders, and hormonal imbalance. Dr. Rehman was selected for the "Research Productivity Award" by the Pakistan Council for Science and Technology (PCST) in 2016.

Introduction to Pharmaceutical Analysis

1

Abstract

Pharmaceutical analysis is a broader term which can be defined in many ways. It is the series of processes that are used for identification, determination, separation, purification, and structure elucidation of the given compound used in the formulation of pharmaceutical products. The components, to which the pharmaceutical analysis is done, are normally active pharmaceutical ingredients, pharmaceutical excipients, contaminants present in pharmaceutical products, or drug metabolites. In pharmaceutical analysis, the samples are typically finished pharmaceutical products, biological samples, impurities, contaminants, and pharmaceutical raw materials. Pharmaceutical analysis can be done using various analytical techniques. This chapter discusses in details the fundamentals of pharmaceutical analysis including its types and its associated important terminologies.

Keywords

Pharmaceutical analysis · Spectroscopy · Chromatography · Analytical techniques

1.1 Introduction

Pharmaceutical analysis is a broader term and there are many ways to define it. It is the process or series of processes that can be used for the identification, determination, separation, purification, and structure elucidation of the given compound used in the formulation of pharmaceutical products. The components, to which the pharmaceutical analysis is done, are normally active pharmaceutical ingredients (APIs), pharmaceutical excipients (disintegrants, binders, surfactants, suspending agents, viscosity increasing agents, polymers, adhesives, lubricants, etc.), contaminants present in pharmaceutical products, or drug metabolites. In pharmaceutical analysis, the samples are typically finished pharmaceutical

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products (tablets, capsules, syrups, creams, lotions, ointments, injections, etc.), biological samples (blood and/or urine, tissue samples that contain one or more ingredients), impurities, contaminants, and pharmaceutical raw materials. Pharmaceutical analysis can be done using various analytical techniques.

1.2 Types of Pharmaceutical Analysis

Pharmaceutical analysis can be classified into the following major types based on the purpose (identification and/or determination) of analysis:

1.2.1 Qualitative Analysis

It is the type of pharmaceutical analysis, in which non-quantifiable method of determination of what constituent or substance is present in an unknown sample or compound. For example, identification of the specific constituent, element, or functional group present in the sample. Identification means to confirm the identity of analyte/s in given sample. Therefore, identification can also be referred to as qualitative analysis. For example, identification of mefenamic acid in Ponstan™ forte tablet is performed with the help of pharmaceutical analysis to make sure that Ponstan™ forte tablet contains exactly the same API as claimed in the label.

1.2.2 Quantitative Analysis

It is the type of pharmaceutical analysis which is intended to measure the exact concentration of the substance of interest in a given sample. In quantitative analysis, as we intend to measure the exact concentration of substance of interest, therefore, it is also referred to as determination. For example, Ponstan™ forte tablet contains 500 mg of mefenamic acid/tablet. So, the quantity of mefenamic acid (500 mg) in individual Ponstan™ forte tablet is determined by the help of pharmaceutical analysis after compression and before releasing into the packing. Determination of exact amount of mefenamic acid in Ponstan™ forte tablet is to make sure that Ponstan™ forte tablet contains correct API that is exactly or close to 500 mg.

1.2.3 Classical Methods for Pharmaceutical Analysis

Following are the most commonly used classical methods that are used in pharmaceutical analysis:

1.2.3.1 Gravimetry

It is the type of classical method of pharmaceutical analysis, in which weight of the sample is determined after the precipitation is occurred.

1.2.3.2 Titrimetry

It is the type of classical method of pharmaceutical analysis, in which volume of the sample is determined after the chemical reaction (neutralization, oxidation, reduction, complex and precipitate formation, etc.) occurred in the sample solution.

1.2.3.3 Volumetry

It is the type of classical method of pharmaceutical analysis, in which the volume of the gas evolved from the sample is determined when the reaction in the sample solution is occurred.

1.2.4 Instrumental Methods for Pharmaceutical Analysis

Following are the most commonly used instrumental methods for pharmaceutical analysis:

1.2.4.1 Optical Methods for Pharmaceutical Analysis

Optical methods are further classified into the following two types:

Absorption of Radiation Methods for Pharmaceutical Analysis

1. UV-VIS spectroscopy.
2. Atomic absorption spectroscopy.
3. IR spectroscopy.

Emission of Radiation Methods for Pharmaceutical Analysis

1. Atomic emission spectroscopy.
2. Molecular fluorescence spectroscopy.
3. Flame spectroscopy.
4. Mass spectrophotometry.
5. NMR spectroscopy.
6. Fluorimetry.
7. Phosphorimetry.

1.2.4.2 Chromatographic Methods for Pharmaceutical Analysis

1. Column chromatography.
2. Thin layer chromatography (TLC).
3. Gas liquid chromatography (GLC).
4. High-performance liquid chromatography (HPLC).
5. Liquid chromatography (LC).
6. Capillary electrophoresis.

1.2.4.3 Electrochemical Methods for Pharmaceutical Analysis

1. Potentiometry.
2. Polarography.

1.2.5 Radiochemical Methods for Pharmaceutical Analysis

1. Ionization method.
2. Liquid scintillation method.
3. Radiometric methods that include radiometric titration, radiochromatography, radioimmunoassay, and isotope dilution method.

1.2.6 Thermal Methods for Pharmaceutical Analysis

1. Differential thermal analysis (DTA).
2. Thermogravimetric analysis (TGA).
3. Differential scanning calorimetry (DSC).

1.3 Where We Do Pharmaceutical Analysis

This is the important question that where can we do the pharmaceutical analysis? Pharmaceutical analysis basically plays a significant role in the development and validation of the processes for good manufacturing practices of high-quality pharmaceutical products. Pharmaceutical analysis is usually carried out in the following fields:

1. Identification and determination of APIs from the bulk drug and/or raw materials.
2. Identification and determination of intermediates of drug during its synthesis.
3. Identification and determination of different stages of drug during the research and development of pharmaceutical products.
4. Identification and determination of in-process quality control of pharmaceutical products during manufacturing.
5. Identification and determination of impurities and degradation products in pharmaceutical products during manufacturing and storage.
6. Identification and determination of API, pharmaceutical excipients, impurities, contaminations, and drug metabolites during drug stability.
7. Identification and determination of metabolites in biological samples containing the pharmaceutical products.
8. Identification and determination of various toxins, poisons, and narcotics in biological fluids.

1.4 Socio-Economic Impact of Pharmaceutical Analysis

High-quality pharmaceutical products exhibit good impact on society by its own way. Pharmaceutical analysis is considered as an important branch of applied analytical chemistry. Pharmaceutical analysis makes sure the high-quality of pharmaceutical products which has direct impact on the economy of the drug therapy

that can also be assessed with respect to the public and financial point of view. Diseased people recover soon after using the high-quality pharmaceutical products as compared to those diseased people who use sub-standard and/or low-quality pharmaceutical products.

1.5 Regulatory Issues for Pharmaceutical Analysis: The Present Situation and Future Trends

Owing to the globalization of pharmaceutical market and sharpening concurrence among the pharmaceutical companies, the term pharmaceutical analysis has become one of the battlefields in struggle which has increased the importance of issues that are directly related to the drug safety and efficacy. To fulfill the legal requirements, analytical techniques for pharmaceutical analysis need to be harmonized. The first step for that purpose was the establishment of European Pharmacopoeia in 1970 which provided the basis for the establishment of national pharmacopoeias by the other countries. The second step was the formation of International Conference on Harmonization (ICH) in 1990 with the aim of harmonizing the efforts of registration agencies, principal pharmacopoeias, and pharmaceutical industries to improve the quality of pharmaceutical products using various analytical techniques for pharmaceutical analysis. The guidelines of ICH have been declared as authoritative worldwide with respect to drug-related issues. By following the ICH-guidelines, pharmaceutical analysis is an important field which increases the safety of drug therapy, but it also acts as a source of inexhaustible intellectual pleasures at the same time.

1.6 Basic Requirements for Pharmaceutical Analysis

To perform the pharmaceutical analysis, the following are the basic and/or fundamental requirements:

1. The first step to perform the pharmaceutical analysis is to define the information you need. The procedures involved in pharmaceutical analysis are generally complex and consist of multiple steps; therefore, it is very important to define the purpose of analysis at this stage to avoid any kind of ambiguity.
2. The second step is the selection of most appropriate methods for pharmaceutical analysis which can be selected by keeping in view the purpose of pharmaceutical analysis.
3. Third step is the collection of required number of samples. This is a very important step. It is crucial to take samples in a representative manner in order to make a precise picture of purpose of analysis. If we talk about the identification and determination of mefenamic acid in Ponstan™ forte tablet, final form of Ponstan™ forte tablet should be taken in a systematic way during the entire time scale of production to give an average of the total production.

The collected samples should be stored at recommended temperature. The sample storage is very crucial because there may be compositional changes in the constituents during storage. Before sampling, the following points should be defined:

a. Purpose of sampling?
b. Type of tests intended to be applied to the samples?
c. Type of pharmaceutical products/materials to be sampled?
d. Are sampling facilities adequate?
e. Are responsibilities of the samplers clear?

Generally, there is no specific rule for sampling, but whenever possible, sample should be obtained from each package or container. In case of single package, contents should be thoroughly mixed and then sampling should be done. In case of large number of sampling, each sample should be labeled properly by mentioning the following information:

a. Name of the sample.
b. Date of sampling.
c. Amount of the sample taken.
d. Name of the sampler, etc.

4. After the collection of the required number of the samples from pharmaceutical product, the next step is the preparation of sample and standard solution to perform pharmaceutical analysis. Samples are normally pretreated in some way and this is called sample preparation. Sample preparation can be very simple or quite complicated, depending on the nature of the sample. For example, in case of Ponstan™ forte tablet, the sample is prepared after pulverization of the tablets, dissolution of the tablets, and filtration of material that has not been dissolved.
5. Next step is to specify the quantities and concentrations for pharmaceutical analysis. For example, quantitative pharmaceutical analysis is performed when analyte is present in a solution. A solution is a homogeneous mixture of two or more substances in which the minor species in a solution is known as solute. The analyte (whose concentration is to be determined) is an example of a solute. The major species in a solution is known as solvent. The amount of analyte (solute) is normally expressed as the concentration which means the amount of solute per volume unit of solution.
6. Once the quantity and concentration have been defined, next step is to do the analysis.
7. Calculate the results and estimate reliability.
8. Convert results to information.
9. Data concerning each analysis should be entered in the laboratory notebook by any way.
10. Fundamental apparatus required for pharmaceutical analysis: Following are some important basic equipment that more or less commonly used during the pharmaceutical analysis of pharmaceutical product depending upon the purpose and nature of pharmaceutical analysis to be performed:

a. *Volumetric flasks*: These are the standard equipment that are used to transfer known volumes of the sample and/or standard solutions with exact concentration and/or volume for pharmaceutical analysis (Fig. 1.1). It contains a narrow neck. There is a mark on the neck of flask for the measurement. The liquid sample is filled up-to this mark to obtain the exact volume. It is available in different sizes to measure the desired volume at a specific temperature and this temperature is usually printed on the volumetric flask.
b. *Graduated cylinders*: These are used to deliver and/or transfer the known volumes of the sample and/or standard solutions during pharmaceutical analysis (Fig. 1.2).
c. *Burettes*: It is a long tube made of glass and there is a tap at the bottom side (Fig. 1.3). There is a graduation scale (usually in milliliter) on the tube that makes it possible to continuously read the volume of the liquid delivered from the burette. They are mostly used in titration where a solution is added gradually into a sample until end point reached where the titration is terminated. Then the consumption of solution can be read off on the burette with high accuracy.
d. *Pipettes*: These are used to deliver and/or transfer the known volumes (usually in milliliters and in some cases in microliters) of the sample and/or standard solutions during pharmaceutical analysis. There are three types of pipettes (Fig. 1.4) that are (1) transfer pipettes, (2) graduated pipettes, and (3) automatic pipettes.
e. *Apparatus for filtration*: Depending on the nature and purpose of pharmaceutical analysis, various types of filtration apparatus and methods can be used during pharmaceutical analysis.
f. *Balances*: Quantitative analysis is primarily based on exact weighing of the analyte which is under consideration. Therefore, weighing should be done with the help of an analytical balance in order to get a maximum possible accuracy. Thus, an analytical balance is considered as a fundamental instrument use in the pharmaceutical laboratory.
g. *Equipment for analysis*: Type of the equipment depends on the purpose of the pharmaceutical analysis.

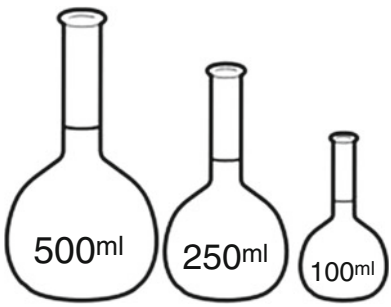

Fig. 1.1 Schematic representation of volumetric flasks having different measuring capacity

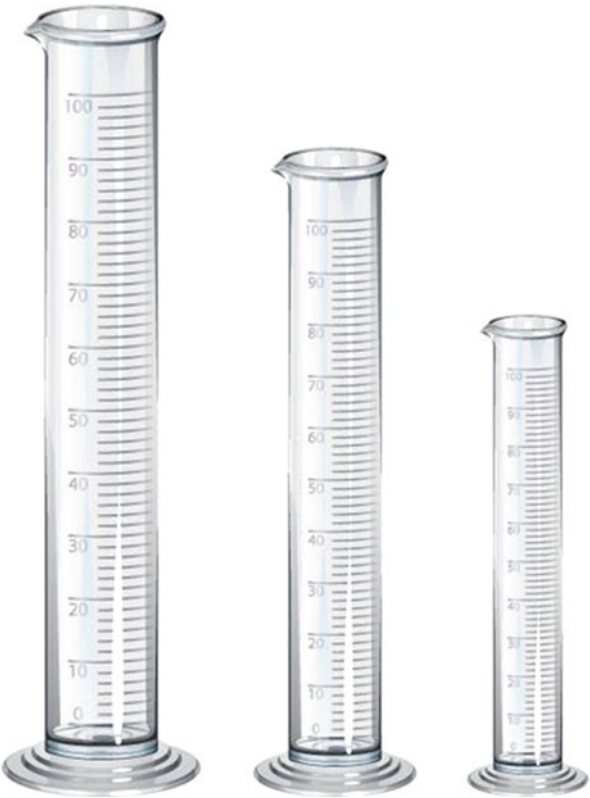

Fig. 1.2 Schematic representation of graduated cylinders having different measuring capacity

1.7 Terms Used in Analytical Techniques

During pharmaceutical analysis, several important terminologies are used. We have described the most important ones here.

1.7.1 Analyte

In pharmaceutical analysis, the substance that is to be identified and/or quantified is known as analyte. The sample contains one or more than one analyte. For example, if we want to determine the exact quantity of mefenamic acid in Ponstan™ forte tablet, then in this analysis, mefenamic acid will be analyte and rest of the tablet material that contains different pharmaceutical excipients will be the sample matrix.

Fig. 1.3 Schematic representation of burette

1.7.2 Analytical Blank

It contains all the reagents or solvents that are used in the pharmaceutical analysis but do not contain any of analyte in it. It is used to check whether the reagents and/or indicators do not contribute to the final results required. It is usually used to calibrate the instruments and/or validate the methods for pharmaceutical analysis. The primary purpose of using analytical blank is to trace sources of artificially induced contamination.

Fig. 1.4 Schematic representation of different types of pipettes

1.7.3 Standard Solution

Standard solution is used in quantitative pharmaceutical analysis. It is defined as the solution which has known amount and/or concentration of the reagent in predetermined volume of the solution. The known amount and/or concentration of the reagent in standard solution is mostly expressed by one of the following ways:

1.7.3.1 Molarity

The number of moles of solute in 1 L of the solution is known as molarity. In pharmaceutical analysis, it is abbreviated as "M."

1.7.3.2 Normality

The number of equivalents of solute in 1 L of the solution is known as normality. It is abbreviated as "N."

In pharmaceutical analysis, the following two types of the standard solutions are used:

1.7.3.3 Primary Standard Solution

Such type of standard solution is made of primary standard substances. Primary standard substance is almost 99.9% pure and is dissolved in the known volume of the solvent. A primary standard solution is typically used to determine the unknown concentration of the analyte. This solution is often used to make the secondary standard solutions. An ideal primary standard substance must have the following properties:

1. It must have high level of purity.
2. It must have low reactivity and high stability.
3. It must not be hygroscopic in nature.
4. It must be non-toxic.

The most common example of primary standard solutions is NaCl solution in which NaCl is used as primary standard substance for silver nitrate ($AgNO_3$) reactions. Zinc is used as primary standard substance for EDTA solutions when it is dissolved in sulfuric acid and/or hydrochloric acid. Potassium hydrogen phthalate is used as primary standard substance for perchloric acid.

1.7.3.4 Secondary Standard Solution

The solution has already been standardized with primary standard solution and then it is used in specific pharmaceutical analysis. Such type of standard solution is known as secondary standard solution. Secondary standard solutions are mostly used to calibrate the equipment and validate the analytical methods. For example, NaOH solution. Once its concentration has been standardized with primary standard solution, it is then used as secondary standard solution. The other examples of secondary standard solutions are oxalic acid and copper sulfate solutions. The secondary standard solutions must have the following features:

1. Its concentration should be stable for longer period of time.
2. It should have the ability to rapidly react with analyte to complete the reaction by simple chemical equation.
3. It should have the ability to produce the sharp end point.

1.7.4 Indicators

In pharmaceutical analysis, indicators play their pivotal role to indicate the completeness of the chemical reaction. They are weak organic acids or bases whose solutions change their color due to change in pH of the solution. In chemical reaction, indicators either produce or disappear specific colors and it is based on the presence of the hydrogen ion concentration in solution. For example, phenolphthalein produces pink color and methyl orange produces red to yellow color.

1.7.5 Calibration of Analytical Method for Pharmaceutical Analysis

It can be defined as the comparison of the value of particular parameter measured by the system under strictly pre-defined conditions with pre-set standard values.

1.7.6 Statistical Analysis

Pharmaceutical analysis does not mean just to perform the experiment in laboratory by dealing and/or handling with chemicals and reagents. Interpretation of the data using various statistical analysis tools enables the analyst to obtain the results that are close to the theoretical values. Statistical analysis ensures the analyst that the method has an appropriate precision and accuracy with minimum errors.

1.7.6.1 Errors

In pharmaceutical analysis, the difference between the true or standard value with observed value is known as error. There is a degree of uncertainty that is associated with every measurement and one can at its best decrease this uncertainty to an acceptable value. While doing pharmaceutical analysis, there may be uncertainty that is related with measurements. It is because of minor errors that may happen in various stages of pharmaceutical analysis. Thus, analytical result is considered as an estimation of the true value and/or content of analyte in the sample under consideration. Therefore, it is important to decrease these uncertainties to a minimum level to ensure that the result is as close to the true value as possible. Error can be calculated by the help of the following formula:

$$\text{Error} = \text{True value} - \text{Observed value}.$$

And %error is calculated by the help of the following formula:

$$\%\text{error} = \frac{\text{True value} - \text{Observed value}}{\text{True value}} \times 100.$$

1.7.6.2 Types of Errors

The possible errors encountered during pharmaceutical analysis are broadly classified into the following two categories:

Determinate Errors

These errors are determinable and presumably can be either avoided or corrected. Such type of errors are also known as systemic errors. These types of errors are known to the pharmaceutical analyst. These have the following types:

Instrumental Errors

These errors are occurred due to the use of the defective and non-calibrated equipment. It is common to all instruments due to their limited accuracy. Calibration of an instrument in one range may not be valid for entire range. For example, a transfer pipette is intended to transfer 10.00 mL. But it is taking up slightly greater volume (10.06 mL). It is because pipette was not calibrated due to which it will give a greater volume (10.06 mL) than expected one (10.00 mL). It will give rise to a systematic error and this error can affect all pharmaceutical analysis during which this uncalibrated pipette has been employed.

Personal Errors

Such type of errors is exclusively caused by the personal mistakes and/or carelessness of the analyst. These can be reduced by experience. For example, mistake of the analyst during reading and/or calculation.

Operative Errors

These errors are physical in nature and occur when an appropriate analytical technique is not followed. Most common examples are transfer of solutions, effervescence during solution, incomplete drying of samples, mathematical errors, etc.

Reagent Errors

These types of errors are dependent on the quality of the reagent used during pharmaceutical analysis. For example, some reagents are supplied in impure form but are pretended to be absolutely pure. When these reagents are used in pharmaceutical analysis, such type of errors occur.

Constant Errors

Sometimes, the errors remain constant throughout the pharmaceutical analysis irrespective of the amount and/or volume of the sample used. For example, in case of titration, the end point appears at 5 mL but for proper visibility of the end point, if we add 0.1 mL, then it would be 5.1 mL instead of 5 mL. If we use 10 mL, then it would be 10.1 and if we use 15 mL, it would be 15.1 mL. Here, 0.1 mL is known as constant error irrespective of the volume used.

Proportional Errors

Proportional increase in the absolute error is known as proportional error. For example, 5.1 mL instead of 5 mL, 10.2 mL instead of 10 mL, 15.3 mL instead of 15 mL. Here, the proportional error is 0.1 mL for 5 mL, 0.2 mL for 10 mL, and 0.3 mL for 15 mL.

Errors in Method

These are inherent in the method and they cannot be changed. These errors occur due to the selection of the wrong method. For example, if the synthesis of one product takes place in 3 h, but we have given only 2 h, then the final product will be defective.

Absolute Error

It is calculated by measuring the difference between a measured value and true value.

Relative Error

It is an absolute error divided by true value.

Indeterminate Errors

These are accidental or random errors that may or may not be known to the analyst. Analyst has no control over such type of errors. It follows random distribution. A mathematical law of probability can be applied to it. Generally, it is easily estimated by measuring the standard deviation for several replicate measurements.

Sources of Errors

Following are the possible sources that may lead to the occurrence of different types of errors:

1. Improper sampling.
2. During sample preparation.
3. Improper and/or careless analysis.
4. Improper function of the equipment.
5. Improper calibration of the equipment.
6. Wrong data and/or improper observation.
7. Wrong calculation.
8. Wrong method selection.
9. Improper handling of materials during transport and/or storage.

1.7.6.3 Limit of Detection

It can be defined as the smallest amount of given analyte that can be detected by using a particular analytical method with known confidence level, but cannot be necessarily quantified as an exact amount. It is abbreviated as LOD.

1.7.6.4 Limit of Quantification

It is defined as the minimum concentration and/or amount of the analyte at which the quantitative pharmaceutical analysis can be performed. It is abbreviated as LOQ. LOQ is actually a range of the analyte concentration where the detector responds the change in the concentration of the analyte.

1.7.6.5 Linearity

It refers to the mathematical relationship that can be graphically represented as a straight line as in two quantities that are directly proportional to each other (Fig. 1.5).

1.7.6.6 Range of Method

During pharmaceutical analysis, analytical method measures the analyte within from minimum up to maximum concentration. It measures both repeatability and reproducibility for a measurement system.

1.7.6.7 Selectivity

The selectivity of a given method reflects to its potential to measure the analyte alone in a given sample consisting of a number of compounds. For examples, the comparison is between the UV spectroscopy and fluorescence spectroscopy. There are various compounds which show UV absorption than there are those that only exhibit strong fluorescence. Therefore, fluorescence is considered as a more selective method in this case.

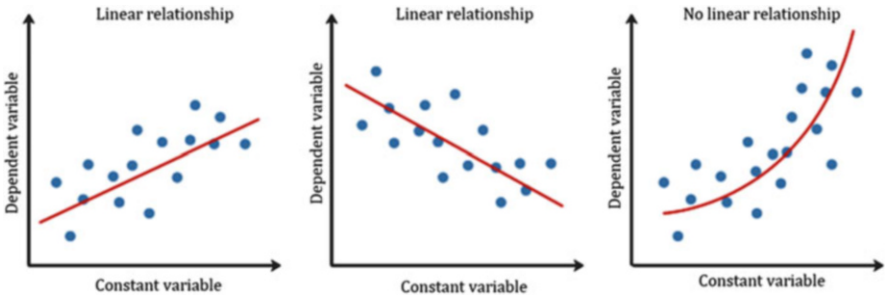

Fig. 1.5 Schematic representation of linear relationship between the constant and dependent variables

1.7.7 Sensitivity

Sensitivity of a particular method shows how responsive it is to a small change in the amount of a given analyte.

1.7.8 Accuracy

During pharmaceutical analysis, the term precision and accuracy is used to describe the quality of analytical measurements. The term accuracy tells how much the analytical results match with the true value. It is generally expressed by the difference between the observed value and true value which is actually an error. The good accuracy means there is the lowest possible difference or error. To find the accuracy of analytical method, true value of the samples must be known, but in case of unknown sample, an experienced analyst can obtain the true value by analyzing the unknown samples with well-defined procedures and/or standard operating procedures. The most suitable way to obtain the true value is to add the known concentration and/or amount of the analyte in a solution to prepare a standard solution (reference solution).

1.7.9 Precision

In quantitative pharmaceutical analysis, analysis of a given sample is performed multiple times to ensure that the obtained results are not affected by the random errors. Variability among the replicate measurements is known as precision. It means that different values are how much close to each other. Precision is usually expressed as standard deviation or relative standard deviation.

1.7.10 Repeatability

It is the term which is carried out in an assay of the particular sample by a single operator or sum total of all the operations carried out by the same operator. For repeatability, conditions that must be followed are same location, measurement procedure, observer, measuring instrument, and environmental conditions. Repetition should be done within short interval of time.

1.7.11 Reproducibility

It is referred as degree to which consistent results are produced, if an experiment is performed multiple times. Reproducibility is confirmed by employing multiple operators or by changing the location or instrument. It has the following two types:

1.7.11.1 Within-Laboratory Reproducibility

Experiment is repeated by the different analyst but in the same laboratory with same equipment.

1.7.11.2 Between-Laboratory Reproducibility

Experiment is repeated by employing different operators, equipment, and laboratory.

1.7.11.3 Dilution

Dilution refers to the process in which concentration of solute is reduced in given sample and it is simply done by adding the more solvent. Serial dilutions are made so that concentration of analyte falls within given range of an instrument. In order to obtain the dilution factor, simply divide the final volume of the solution by initial volume of the solution. For example, mix 0.1 mL of solution with 9.9 mL of solvent. The final volume of the solution is 10 mL. Divide final volume (10 mL) by initial volume (0.1 mL), dilution factor 100 is obtained and it is written as 1:100 dilution.

1.7.11.4 Analysis of Variance

It is abbreviated as ANOVA. It has a significant role in the field of pharmaceutical analysis. ANOVA can be calculated by dividing the total variance present in experimental data over several factors and/or components. ANOVA is a parametric test which requires sum of the squares of the data and treatment sum of squares.

1.8 Properties of Drug

Pharmaceutical analysis of drug is totally dependent on the nature and/or properties of the drug to be analyzed. Drug that is to be analyzed possesses two types of properties that are as follows:

1.8.1 Physical Properties of Drugs

Physical properties are measured or observed without any change in the matter composition. Physical properties include appearance, odor, color, melting point, boiling point, solubility, density, polarity, opacity, viscosity, and many others. These properties play an important role in the design of analytical methods for pharmaceutical analysis.

1.8.2 Chemical Properties of Drugs

Chemical properties are observed whenever a substance undergoes a chemical change. Ionization, functional groups, partition coefficient, pH, degradation and stereochemistry, heat of combustion, and enthalpy change are the important chemical properties that should be considered during the selection of an appropriate analytical method for pharmaceutical analysis.

1.9 Standard Operating Procedures (SOP)/Protocols

SOP is a set of step-by-step instructions complied by an organization to help analyst carry out routine analysis in the laboratory. Pharmaceutical analysis is totally based on these SOPs and/or protocols. First, the SOPs and/or protocols are developed in laboratories by experts. SOPs must be repeatable and reproducible. SOPs and/or protocols must be in accordance with the guidelines of ICH and pharmacopoeias. SOPs help the analyst to perform pharmaceutical analysis and also able the analyst to avoid any kind of possible errors.

1.10 Applications of Pharmaceutical Analysis

Pharmaceutical analysis is a broader field and its applications are vast which are as follows:

1. Identification and determination of APIs in bulk drug and/or raw material and finished dosage forms of the pharmaceutical products.
2. Identification and determination of impurities in bulk drug and/or raw material and finished dosage forms of the pharmaceutical products.
3. Identification and determination of contaminants in bulk drug and/or raw material and finished dosage forms of the pharmaceutical products.
4. Identification and determination of various drug metabolites and/or intermediates in biological samples.

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Introduction to Spectrophotometric Techniques

2

Abstract

Spectrometric techniques are used to measure the interaction of different frequency components of electromagnetic radiations (EMR) with that of matter. After interaction with matter, these radiations are absorbed by the matter. It is not possible that we look at the matter instead, we observe the interaction of light with different degrees of freedom of matter and/or substance. This chapter describes the basics of spectrophotometry and various types of spectrophotometric techniques. Spectrophotometry is a key method that is frequently used for identification and quantification of raw materials and pharmaceutical products.

Keywords

Electromagnetic radiations · Frequency · Electronic energy level · Spectrophotometer · Types of spectrophotometric techniques

2.1 Introduction

Spectrometric techniques are used to measure the interaction of different frequency components of electromagnetic radiations (EMR) with that of matter. Electromagnetic radiations interact with matter at specific energy levels. After interaction with matter, these radiations are absorbed then atoms/molecules of analyte present in sample move from one energy state (usually ground state having low energy level) to another energy state (usually excited stated having high energy level). It is not possible that we look at the matter and/or substance but its ghost when the matter and/or substance interacts with light. Instead, we observe the interaction of light with different degrees of freedom of matter and/or substance. In spectrophotometry, we either measure the absorbance or emission of EMR after its interaction with matter. Interaction of EMR with matter is directly dependent on the energy of radiation. Spectrophotometry is used to understand how different frequency components of EMR interact with matter and how we can use this information to understand

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the quantity of analyte present in sample. Broadly speaking, spectrophotometry is basically the set of tools that can be used together in different ways to understand the chemical properties and/or nature of the analyte present in sample.

2.1.1 Photometry

It is the branch of science that deals with the measurement of light. Photometer is a tool that is used for the measurement of intensity of light. It is the measurement of the amount of luminous light (either absorbed or emitted) falling on the surface of analyte present in sample from a source of radiation.

2.1.2 Spectrophotometry

It is the phenomenon of measurement of the intensity of light at selected wavelengths. This method depends on the light absorbing and/or emission property of either the analyte or a derivative of analyte being analyzed. Spectrophotometry is used in pharmaceutical analysis for the identification of the analyte through the spectrum that is absorbed and emitted by an analyte present in the sample solution.

2.2 Spectrum

It is defined as the measurement of response as a function of wavelength or more precisely the frequency.

2.3 Electromagnetic Radiations

Electromagnetic radiations (EMR) contain discrete energy packets. These discrete energy packets are known as photons. A photon contains an oscillating magnetic field and an oscillating electric field (Fig. 2.1). Both of these fields fall perpendicular to one another.

2.3.1 Frequency (ν)

Frequency is defined as the number of oscillations produced by an electrical field radiation per second. Hertz (Hz) is the unit used for frequency (1 Hz = 1 cycle per second).

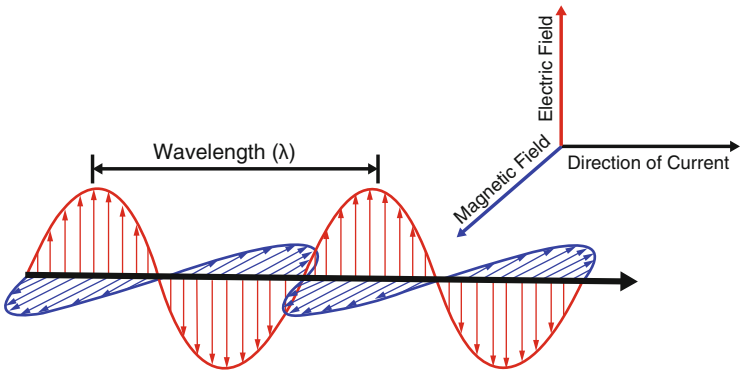

Fig. 2.1 Schematic representation of levels of electromagnetic radiations

2.3.2 Wavelength (λ)

It is the measurement of distance that exists between two adjacent parts of a wave present in the same phase or the distance present between two adjacent troughs or crest.

2.3.3 Levels of Electromagnetic Radiations

In EMR, three levels exist which are as follows:

2.3.3.1 Electronic Energy Level

Molecules exist in the lowest energy level (E_0) at room temperature that is known as ground state. Whenever, the EMR are absorbed by the molecules, valence electrons are promoted to next higher energy state abbreviated as E_1 that is also known as excited state. This shifting of electrons from lower energy level (ground state) to higher energy level (excited state) by absorbing the energy in the form of EMR is known as electronic transition and the difference is calculated as: $DE = E_1 - E_o$.

2.3.3.2 Vibrational Energy Level

These are the less energy levels than electronic energy levels. The difference between these energy levels is comparatively small such as 0.01–10 kcal/mole. For example, when infrared (IR) radiations are absorbed by the molecules, they are excited from lower vibrational energy level to higher vibrational energy level and start vibrating with relatively higher amplitude.

2.3.3.3 Rotational Energy Level

Rotational energy levels are quantized and discrete. The difference between these levels is relatively smaller as compared to that of vibrational energy levels. Overall, order of different energy level is represented as follows:

$$\Delta E_{rotational} < \Delta E_{vibrational} < \Delta E_{electronic}.$$

2.4 Principle of Spectroscopy

The principle of spectroscopy is based upon the measurement of spectrum of given analyte present in sample having either atoms or molecules. Spectrum consists of the graph of intensity radiations either absorbed or emitted by the sample versus wavelength (λ) or frequency (ν). Spectrometer is a device which is used to measure the spectrum of analyte. It is more advanced instrument than colorimeter. It is because there are filters present in colorimeter which allow wide range of wavelengths of radiation to pass through, whereas in spectrophotometer, grating and/or prism is utilized in order to split the light beam into multiple wavelengths. With the help of selective mechanism, specific wavelength of EMR may interact with sample solution.

2.5 Spectrophotometer

This is an optical instrument that is used to measure the intensity of EMR relative to the specific wavelength. It is useful for the measurement of analyte concentration present in sample solution by measuring the amount of EMR absorbed and/or emitted by the sample solution. The sample to be analyzed by spectrophotometer must be in solution form and the solvent that contains analyte must be optically transparent in specific wavelength region.

2.5.1 Components of Spectrophotometer

Following are the four major components of spectrophotometer:

1. *Light source*: It provides the polychromatic light to the monochromator which diffracts the light. The sources of light depend on the type of spectrophotometric technique. For UV-radiation, the most common sources of light are hydrogen lamp and the deuterium lamp. For visible radiation, the most common sources are tungsten filament and carbon arc. For IR-radiation, the most common sources are Nernst Glower and Global.
2. *Monochromator*: It splits polychromatic light into individual wavelengths and separates these individual wavelengths into narrow bands.
3. *Cuvette and/or sample holder*: It holds the sample and allows the specific wavelength to pass through itself. Cuvettes are transparent in nature and are made of ordinary glass or quartz material.
4. *Photosensitive detector*: Detector must be highly sensitive and long-term stable that have the ability to detect the low level of the radiant energy.

Fig. 2.2 Schematic representation of single-beam spectrophotometer

5. *Read-out device*: It has the ability to interpret the signals received from the detector.

According to the number of beams used, spectrophotometer has the following two types:

2.5.2 Single-Beam Spectrophotometer

Single beam passes through sample present in cuvette (Fig. 2.2). At first, the spectrophotometer is standardized by putting a reference solution in the cuvette, and the resulting absorbance is subtracted from absorbance of sample solution in order to remove solvent effect. Single-beam spectrophotometer follows Beer–Lambert law to determine the unknown concentration of the analyte.

2.5.2.1 Advantages

1. Less expensive.
2. High sensitivity due to non-splitting of the light source.

2.5.2.2 Disadvantages

Single-beam spectrophotometer is not suitable due to the lack of the compensation of the following disturbances:

1. Analysis is time consuming as it has single cell. At first, the reference solution is loaded to record the reading, then cuvette is washed and sample solution is loaded to record the recording.
2. Electronic circuit fluctuations.
3. Voltage fluctuations.
4. Mechanical component's instability.
5. Drift in energy of light sources.

2.5.3 Double-Beam Spectrophotometer

A double-beam spectrophotometer is used for the comparison of the light intensity between two light paths, one beam passes through a reference and the other from the test sample (Fig. 2.3).

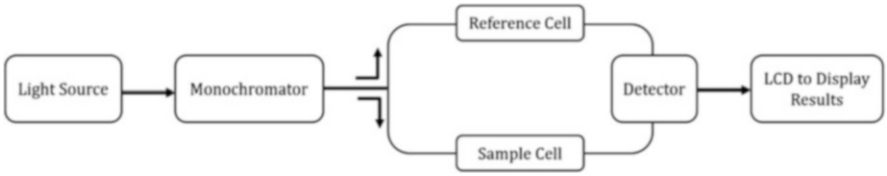

Fig. 2.3 Schematic representation of double-beam spectrophotometer

2.5.3.1 Advantages

1. It offers better detection than single-beam spectrophotometer.
2. Instability factors, lamp drift, voltage fluctuations, and stray light do not affect the measurement of light as these factors influence the measurement of results in single-beam spectrophotometer.

According to the range and type of EMR used, it has the following types:

1. UV-VIS spectrophotometer.
2. IR spectrophotometer.
3. Atomic absorption spectrophotometer (AAS).
4. Atomic fluorescence spectrophotometer (AFS).
5. X-ray fluorescence spectrophotometer (XFS).
6. Mass spectrophotometer (MS).

2.5.4 Types of Spectrophotometric Techniques

Types of the spectrophotometric technique depend on the quantitative pharmaceutical analysis of analyte which is measured by the interaction of different frequency components of EMR with analyte. Mostly, the spectrophotometric techniques can be classified into the following major types:

2.5.5 Absorption Spectroscopy

In this spectrophotometric technique, we measure the amount of EMR that is being absorbed by the sample (Fig. 2.4). The examples are UV-VIS spectroscopy, atomic absorption spectroscopy, infra-red spectroscopy, and nuclear magnetic resonance spectroscopy.

2.5.6 Emission Spectroscopy

In this spectrophotometric technique, when EMR interacts with sample, the sample emits the radiations of specific wavelength which are then detected to predict the

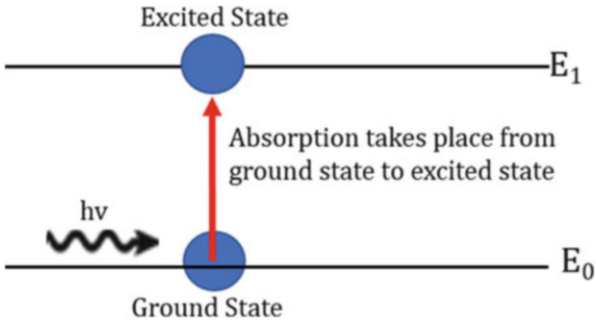

Fig. 2.4 Schematic representation of absorption of light in atomic absorption spectroscopy

amount of the sample. In emission spectrophotometric technique, the sample first absorbs the EMR and then emits the light of specific wavelength. The examples are atomic emission spectroscopy, fluorescence emission spectroscopy.

2.5.7 Scattering Spectroscopy

In this technique, the substance scatters the EMR at specific wavelength and polarization angles. Scattering of light gives the information regarding the molecular structure. The scattering phenomenon is much faster than the absorption and/or emission phenomenon. One of the most important examples of light scattering spectroscopy is Raman spectroscopy. It is used for qualitative purpose. It is helpful for the identification of molecules and chemical bonds.

2.6 Applications

Spectroscopic techniques have wide range of applications in the field of pharmaceutical analysis. The most important are as follows:

2.6.1 Quantitative Analysis

Spectrophotometer is widely used in the quantitative pharmaceutical analysis. In quantitative pharmaceutical analysis, the unknown concentration of analyte may be determined with the help of absorption spectrophotometry because most of the biological organic compounds have the ability to absorb EMR in UV-VIS region. For example, nucleic acids and proteins absorb at 254 nm and 280 nm, respectively.

2.6.2 Qualitative Analysis

UV-VIS spectrophotometer is used to identify the compounds both not only in pure form but also in biological preparations. Identification of compounds can be done by plotting the absorption spectrum of the analyte because the absorption at specific wavelength gives some hints to the structure of the compound. For example, absorption at 220–280 nm indicates that the analyte may be aliphatic, alicyclic hydrocarbons or their derivatives. Absorption at 250–330 nm indicates that the analyte may contain more than two conjugated double bonds.

2.6.3 Enzyme Assay

Enzyme assay is basically performed with the help of spectrophotometer. In this assay, the substrate absorbs light in UV region. For example, lactate dehydrogenase (LDH) is an enzyme that converts lactate to pyruvate with the help of co-enzyme NAD^+. In this reaction, LDH transfer the electrons from lactate to NAD^+ as shown in the following equation.

$$\text{Lactate} + \text{NAD}^+ \leftrightarrow \text{Pyruvate} + \text{NADH} + \text{H}^+.$$

In spectrophotometer, NADH absorbs radiations at the wavelength of 340 nm in UV range, while NAD^+ does not absorb radiation at this wavelength.

Another example of enzyme assay is pyruvate kinase that converts phosphoenol-pyruvate to pyruvate and produces one molecule of ATP. Then pyruvate is converted into lactate.

$$\text{Phosphoenolpyruvate} + \text{ADP} \leftrightarrow \text{Pyruvate} + \text{ATP}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \leftrightarrow \text{Lactate} + \text{NAD}^+.$$

In this reaction, NADH is oxidized to NAD^+ by converting pyruvate into lactate. As NAD^+ does not absorb radiations at 340 nm in UV range, the absorbance decreases which indicate the conversion of pyruvate to lactate.

2.6.4 Molecular Weight Determination

Spectrophotometer can help to determine the molecular weight of the analyte. Spectrophotometer can only determine the molecular weight of the small compounds.

2.6.5 Physicochemical Properties

Spectrophotometer can be used to determine the following physicochemical properties of the analyte:

1. Heats of formation for molecular addition in compounds and/or complexes.
2. Determination of the empirical formula of the substrates.
3. Dissociation constants of acids and bases.
4. Determination of the rate of reactions of the substrates.
5. Quantitative and qualitative determination of the complexes.
6. Hydration equilibration of carbonyl compounds.

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Ultraviolet-Visible (UV-VIS) Spectroscopy

3

Abstract

UV-VIS spectroscopy is considered as the most important spectrophotometric technique that is most widely used for the analysis of variety of compounds. This technique works on the basis of the measurement of interaction of electromagnetic radiations (EMR) with matter at particular wavelength. In this chapter, we have briefly described the UV-VIS spectroscopy by covering the fundamentals of UV-VIS spectroscopy, origin of spectra along with the types of electronic transitions. We have also described the effect of solvents on the absorption spectra of analyte. For proper working of UV-VIS spectroscopy and to get accurate results, it is very important to understand the components of UV-VIS spectroscopy and their individual role in the proper functioning of UV-VIS spectrophotometer. In UV-VIS spectroscopy, absorption of light is the basic phenomenon and we have also described the various absorbance laws on which UV-VIS spectroscopy works. At the end of this chapter, we have also discussed the various terms that are used in this spectroscopy along with the diverse applications of this analytical technique.

Keywords

Electronic transitions · Absorption spectra · Absorbance laws · Beer–Lambert law · Chromophore

3.1 Introduction

UV-VIS spectroscopy is considered as the oldest analytical technique that can be defined as the spectrophotometric technique which is used to measure the intensity of light in UV (10–400 nm) and VIS (400–800 nm) regions as a function of wavelength. The wavelengths of UV and VIS radiations are usually expressed in nanometers (nm). The analyte absorbs the light of specific wavelength (UV and VIS only) and the amount of radiation absorbed by the analyte is measured. The spectrum

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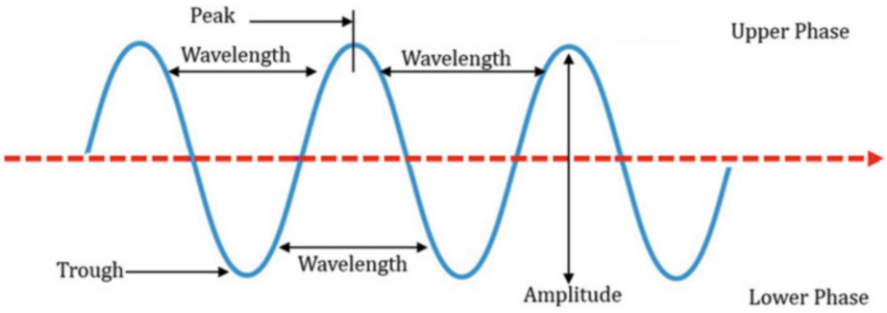


Fig. 3.1 Schematic representation of wave phenomenon

produced after the absorption of UV-VIS light results from the interaction of EMR in UV-VIS region with the analyte. It forms the basis to analyze a variety of substances like organic, inorganic, biochemical, and pharmaceutical compounds. In UV-VIS spectroscopy, absorption of radiations occurs at electronic energy levels (one of the three basic energy levels, i.e., electronic, vibrational, and rotational energy levels) of molecules; therefore, this technique is also known as electronic spectroscopy.

Electromagnetic waves (Fig. 3.1) are described in terms of frequency (v), wavelength (λ), and distance present between the two peaks. The wavelength can be defined as the distance between the two adjacent parts of a wave existing in the same phase, i.e., the distance between the adjacent troughs or crest (Fig. 3.1). Frequency can be defined as the number of times electrical field radiation oscillates in 1 s. Hertz (Hz) is the unit used for frequency and 1 Hz = 1 cycle per second.

The arithmetic relationship among the speed of light (c), the wavelength (λ), and frequency (ν) can be written as:

$$c = v\lambda.$$

According to the laws of quantum mechanics, photon is subjected to energy, so that the above equation may be considered as:

$$E = h\nu = \frac{hc}{\lambda},$$

where E represents the radiation energy, v is the frequency, h is the Planck's constant, c is speed of light, and λ is the wavelength of light that is going to be absorbed.

3.2 Principle

UV-VIS spectroscopy works on the basis of the absorption phenomenon of light and the amount of absorbed light is directly proportional to the amount of analyte present in a sample solution. As the concentration of analyte is increased, absorption

of light is also increased linearly, whereas the transmission of light is decreased exponentially. In UV-VIS region, the absorption of radiation depends on the electronic configuration of the absorbing species like atoms, molecules, ions, or complexes.

An electronic energy level consists of various vibrational energy levels, whereas a single vibrational energy level consists of various rotational energy levels. When a photon interacts with a molecule, it may induce a transition in electronic energy levels if energy provided by the photon matches with energy difference in these levels. The amount of radiations absorbed by the analyte is measured and plotted against the wavelength of EMR in order to obtain the spectrum. Thus, a typical UV-VIS spectrum is a plot of wavelength or frequency versus the intensity of absorption.

3.3 Theory

In a molecule, electrons are associated with more than one nucleus and they are involved in making bonds among the various atoms. These bonding electrons are susceptible to transitions in various energy levels under the provocation of appropriate radiations. The details of various levels of electronic transitions have been briefly described in the following sub-sections.

3.4 Electronic Transitions

The absorption of electronic energy levels (UV-VIS radiations) by an organic molecule is associated with the excitation of valence electrons from ground state to excited state (Fig. 3.2). After absorption of energy, the electronic transitions are generally occurred from the excited state that contain the highest molecular orbital (this phenomenon is known as antibonding). The wavelength of the absorbed radiation depends upon the difference of energy between the orbital (E_2) which is originally occupied by the electron (ground state) and the orbital to which it is promoted (E_1).

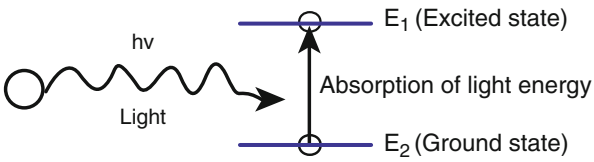


Fig. 3.2 Schematic representation of electronic energy levels

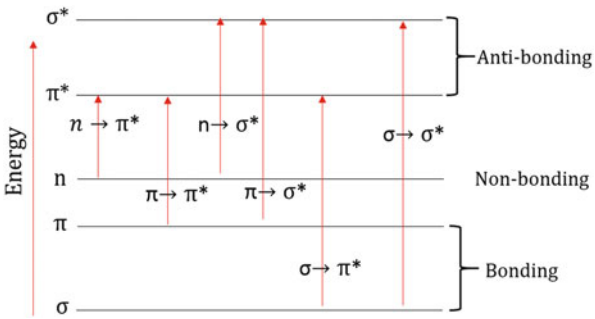


Fig. 3.3 Schematic representation of various levels of electronic transitions

3.4.1 Types of Electronic Transitions

Electrons in an organic molecule are involved in bonding as strong (σ bond), weak (π bond) or present as non-bonding lone pair. Based on the nature of the bond present in organic molecule, absorption of electronic transitions that are associated with the absorption of UV-VIS radiations are of following four types (Fig. 3.3).

3.4.1.1 $\sigma \rightarrow \sigma*$ Electronic Transition

These electrons are held firmly within the molecule. Large amount of energy is required for the transition of σ (bond) to σ∗ (antibonding) that is corresponding to the far UV region with the range of 120–200 nm. This type of transition is present in saturated hydrocarbons such as CH_3–CH_3 that contain only strongly bound σ electrons and the excitation of these electrons from σ orbital to σ∗ orbital requires large amount of energy. Methane (CH_4) shows $\sigma \rightarrow \sigma*$ electronic transition at λ_{max} at 122 nm.

3.4.1.2 $\pi \rightarrow \pi*$ Electronic Transition

This type of transition is present in the compounds that contain double and/or triple bonds or aromatic rings and the excitation of electrons requires less energy as compared to the $\sigma \rightarrow \sigma*$ electronic transition which indicates that $\pi \rightarrow \pi*$ electronic transition occurs at larger wavelength (160–190 nm). For example, ethylene shows absorption at 171 nm, whereas a conjugated unsaturated bond such as 1,3-butadiene absorbs at a much higher wavelength (217 nm). Acetone $(CH_3)_2C{=}O$ and benzene (C_6H_6) show $\pi \rightarrow \pi*$ electronic transition at λ_{max} of 122 nm and 255 nm, respectively.

3.4.1.3 $n \rightarrow \sigma*$ Electronic Transition

The excitation of electrons in an unshared pair form on an atom to an antibonding sigma orbital is called as $n \rightarrow \sigma*$ electronic transition. This type of transition is present in saturated molecules such as halides, ethers, alcohols, etc. The excitation in $n \rightarrow \pi*$ electronic transition involves the excitation of an electron from n orbital (non-bonding) of the heteroatom to an antibonding sigma orbital (σ∗) of the molecule. The energy required for the transition of electrons from n orbital (non-bonding)

to antibonding sigma orbital (σ∗) is less than that of the σ → σ∗ electronic transition which results in the absorption of longer wavelength of the UV-VIS region (between 150 and 250 nm). For example, methyl alcohol shows n → σ∗ electronic transition at 183 nm, whereas trimethylamine at 227 nm. Trimethylamine does not show n → σ∗ electronic transition in aqueous acid because the protonated amine does not contain non-bonding electrons. Chloromethane (CH_3Cl) and benzene (C_6H_6) show n → σ∗ electronic transition at λ_{max} of 173 nm and 277 nm, respectively.

3.4.1.4 n → π∗ Electronic Transition

In this type of transition, the unshared pair electrons on heteroatoms are excited to antibonding pi star (π∗) orbital and in this orbital, the non-bonding electrons are held loosely; therefore, the transition requires longer wavelengths. This type of transition occurs in compounds that contain double bonds involving heteroatoms having unshared pair of electrons such as C=O, C=S, N=O, etc. For example, ketones and aldehydes exhibit two bands at 180–200 nm (π → π∗) and 280 nm (n → π∗). n → π∗ electronic transition is not strong as the electrons in non-bonding n orbital are situated at perpendicular to the plant of the π bond and hence the chance of the jump of an electron from n orbital to pi star orbital (π∗) becomes low.

3.5 Origin of Absorption Spectra

The absorption of UV-VIS radiations is based on the electrons in molecules that may be promoted to higher energy levels after the absorption of energy. The electrons present in molecules can be categorized into the following types:

3.5.1 σ-Electrons

These electrons are involved in making single covalent bond and high energy radiation is required in order to excite them.

3.5.2 π-Electrons

These electrons are involved in making either double or triple bond and relatively low energy radiation is required in order to excite them.

3.5.3 n-Electrons

These types of electrons are attached with nitrogen, oxygen, and chlorine as a lone pair of electrons and also known as non-bonding electrons. These electrons require less energy for excitation as compared to both σ and π electrons.

3.6 Effect of Solvent on Absorption Spectra

Nature of the solvent (either polar or non-polar) in which the analyte is present may interfere with the absorption spectra of analyte. Saturated hydrocarbons are non-polar solvents and if are highly pure, they do not interfere with the solute molecules neither in ground state nor in excited state. The absorption spectra of analyte in such type of solvent are similar to that in pure gaseous state. Polar solvents like water or alcohols may stabilize or destabilize orbitals of molecules both in ground and excited state. Solvents (both polar and non-polar) play distinguished role in the absorption spectra determined by UV-VIS spectroscopy. Therefore, the solvent should not interact with the analyte and should not interfere with the absorption spectra of analyte.

3.7 Effect of Electronic Transitions on Absorption Spectra

Despite the nature of the solvent, electronic transition state may also affect the spectrum of the analyte. Among the four types of electronic transitions ($n \rightarrow \pi*$, $n \rightarrow \sigma*$, $\pi \rightarrow \pi*$, and $\sigma \rightarrow \sigma*$), $n \rightarrow \pi*$ and $\pi \rightarrow \pi*$ electronic transitions affect the absorption spectrum of analyte.

3.7.1 Effect of $\pi \rightarrow \pi*$ Electronic Transitions on Absorption Spectra

In $\pi \rightarrow \pi*$ transition, the excited state shows more polarity as compared to that in the ground state, whereas the dipole–dipole interaction with solvent decreases the energy of electrons at excited state as compared to that in ground state. Thus, polar solvents decease the $\pi \rightarrow \pi*$ electronic transition energy and the absorption maxima of the analyte appears ~10–20 nm (red shift or bathochromic shift) when the polar solvent is changed from hexane to ethanol.

3.7.2 Effect of $n \rightarrow \pi*$ Electronic Transitions on Absorption Spectra

In $n \rightarrow \pi*$ electronic transitions, polar solvents with hydrogen bonds contain more readily polar molecules at ground state as compared to that of their excited state and hence in polar solvents, the electronic transition energy is increased.

3.8 Components of UV-VIS Spectrophotometer

Following are the main components of UV-VIS spectrophotometer:

3.8.1 Light Sources

It provides a sufficient light in the form of polychromatic light that is appropriate for marking the measurements. Usually, light source provides polychromatic light over a wide range of spectrum (Fig. 3.4). In UV-VIS spectrophotometer, two types of light sources are used. For UV sources, deuterium, hydrogen, tungsten, mercury, and xenon lamps are used, whereas for VIS sources, tungsten, mercury vapor, and carbon lamps are used.

3.8.2 Monochromator

It is a device that receives the polychromatic light as input from a lamp and provides output in the form of monochromatic light (Fig. 3.5). This device is used to disperse the radiations of polychromatic light according to the wavelength.

3.8.2.1 Components of Monochromator

Monochromator consists of three essential components (Fig. 3.6) that are collectively involved to disperse polychromatic light into monochromatic light. These are entrance slit, dispersing device, and exit slit.

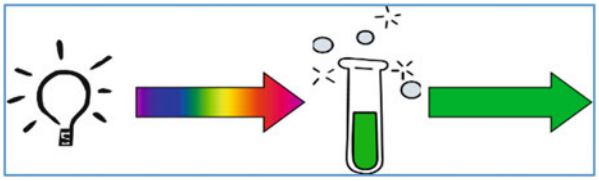


Fig. 3.4 Schematic representation of light source

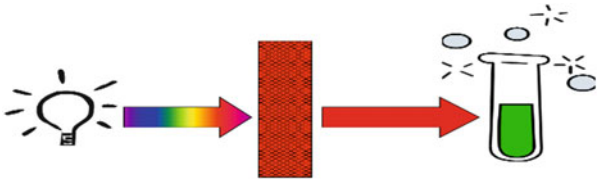


Fig. 3.5 Schematic representation of monochromatic light

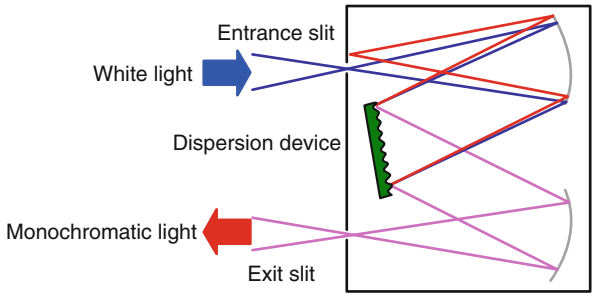


Fig. 3.6 Schematic representation of components of monochromator

Entrance Slit
It sharply defines the incoming polychromatic beam of light and imparts it on dispersing element.

Dispersing Device
It is a special plate that contains hundreds of parallel grooved lines. These lines are used to separate the polychromatic light (white light) into visible light spectrum. It disperses the polychromatic light that is coming from the entrance slit into its component wavelengths with the help of focusing lens. Dispersing element, such as prism or grating is usually made up of quartz, fused silica, or glass.

Exit Slit
Exit slit allows the minimal wavelength together with the band of wavelengths on either side. The position of exit slit is not fixed and it is mostly needed to adjust it by rotation to vary the minimal wavelength passing through the exit slit.

3.8.2.2 Working of Monochromator

Polychromatic light is collimated and hits the dispersing element at an angle and splits into its component wavelength by prism or grating after entering monochromator through the entrance slit. By rotating the dispersing element and exit slit, radiation of required particular wavelength is obtained from monochromator via exit slit.

3.8.3 Sample Device/Cuvette

Cuvettes are available in various forms for UV-VIS region (Fig. 3.7). They are specifically designed to hold the sample for spectrophotometric analysis. They vary with respect to shape, size, path length, and transmission characteristics of required wavelength. Cuvettes are made up of plastic, glass, or optical grade quartz that does

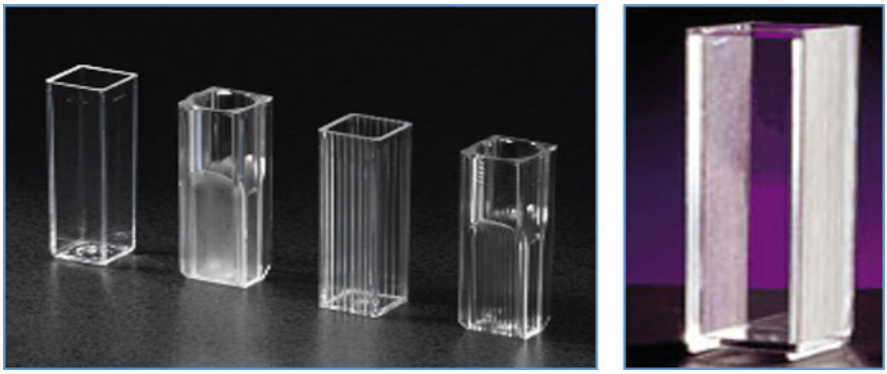


Fig. 3.7 Schematic representation of sample device or cuvette

not absorb the λ range of interest. Before to do the analysis, cuvette should be cleaned thoroughly and they should be free from all types of impurities that may alter the spectroscopic reading.

3.8.4 Detector

It is a device that can be used for the measurement of the amount of light passing through the sample and convert the light signals (coming from the sample) into the electrical signals. In order to detect the radiation in UV-VIS spectroscopy, three kinds of photosensitive devices are used which are as follows:

3.8.4.1 Photovoltaic Cells or Barrier Layer Cell

It contains metallic base plate such as iron or aluminum that acts as an electrode. Semiconductor, like silicon or germanium, is deposited on its surface. Over it, thin layer made of gold or silver is present that acts as second collector tube. When light falls over Se, electrons are generated at Se-Ag surface and these electrons are collected by the silver. Excess of electrons on silver surface produces the voltage difference between the silver and base of cell.

3.8.4.2 Phototubes or Photoemissive Tubes

It contains an evacuated glass tube. A light sensitive cathode is present inside it. Cathode is coated from inner side with light sensitive material like silver oxide and potassium oxide. When cathode is exposed to radiation, there is the emission of photoelectrons and they are collected by anode and are returned through the external circuit. Current is amplified and recorded by this process.

3.8.4.3 Photomultiplier Tubes

It is the most commonly used type of detector. It contains a photoemissive cathode. It contains numerous dynodes (emit numerous electrons in response to each electron hitting them) and an anode. A photon strikes to the cathode after entering the tube and it causes emission of numerous electrons that accelerate to the first dynode. First dynode is 90 V more positive as compared to that of cathode, several electrons emitted for each incident electron are further accelerated and more electrons are produced by this process, eventually electrons are collected at anode. By this way, each original photon produces 106–107 electrons, resulting current is amplified and recorded.

3.8.5 Recorder

Spectrometer provides the signals to the recorder and intensity of signals depends upon light absorption by the analyte at a particular wavelength. These signals are amplified by amplifier and elaborated by the personal computer. Digital read-out

devices like light emitting diode or liquid crystal display (LCD) are used for clarity purpose that removes the ambiguity.

3.9 Types of UV-VIS Spectrophotometer

Design of spectrophotometer is based on the fundamental principle of light absorption by absorbing species. Based on the number of cuvettes and beams used, UV-VIS spectrophotometer is classified into the following two types:

3.9.1 Single-Beam UV-VIS Spectrophotometer

In this type of spectrophotometer, single beam and/or cuvette is used for the analysis (Fig. 3.8). In single-beam UV-VIS spectrophotometer, a single light beam passes across the cuvette. The spectrophotometer is calibrated by putting the reference and/or standard solution in the cuvette and then the resulting value of absorbance is subtracted from sample measurements in order to remove the effects produced by the solvent and cell. This type of spectrophotometer is appropriate for applications in the wavelength range of 190–1100 nm. Various types of samples including nucleic acid, proteins, and a number of organic molecules are often analyzed in this region. Whereas, the visible region of the electromagnetic spectrum is from 340 to 750 nm, and this region is appropriate for the analysis of colored samples.

3.9.2 Double-Beam UV-VIS Spectrophotometer

In this type of spectrophotometer, two beams and/or cuvettes are used for the analysis (Fig. 3.9). The light beam coming from the source of light is split into the sample and reference beam with the help of mechanical chopper. The role of reference beam is to monitor the intensity of light energy while the sample beam

Fig. 3.8 Schematic representation of single-beam spectrophotometer

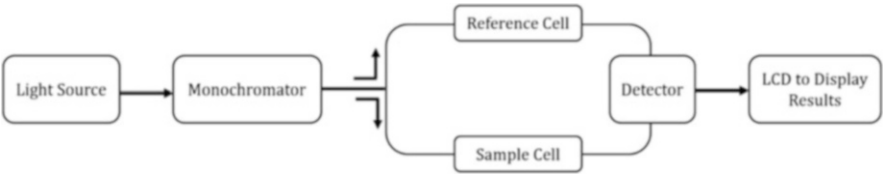


Fig. 3.9 Schematic representation of double-beam spectrophotometer

shows absorption of light from the sample. The value of observed absorbance which is the ratio of the sample and reference beam is recombined before it enters into the monochromator. This arrangement compensates the effects of light reference and sample beam due to drift in lamp intensity, electronic and mechanical fluctuations which affect the reference and sample beams equally. Although, double-beam UV-VIS spectrophotometers are bit complicated as compared to that of single beam, still they have some advantages which are as follows:

1. The sample and reference run simultaneously. There is no need to adjust zero at each wavelength or replace the blank with sample.
2. The ratio of the powers of reference and sample is constantly obtained.
3. The scanning speed is rapid over a wide range of wavelength and digital read-out device is used to record the electrical signals.

3.10 Absorbance Laws

There are basically three laws for spectroscopy that describe the absorbance of light through a material. The details of these laws have been described below.

3.10.1 Beer's Law

It can be described as the beam intensity of the monochromatic light is decreased exponentially when the concentration of analyte increases arithmetically (Fig. 3.10). In quantitative analysis, primarily concerned with solutions, the effect of concentration of the colored constituent in solution depends upon the light absorption or transmission. It can be expressed as:

$$I = Ie^{-klc}.$$

3.10.1.1 Beer Derivation

In 1852, Beer described the following relationship between the concentration of a solution and absorbance of light:

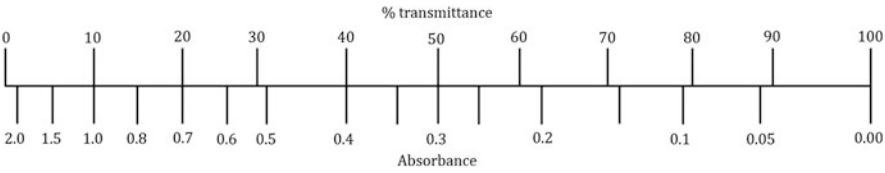


Fig. 3.10 Schematic representation of % transmittance vs. absorbance

$$\log \frac{I_0}{I_T} = \frac{k^n c}{2.303} \quad \text{or} \quad A = \frac{k^n c}{2.303}$$

where c is the concentration of analyte in sample solution, k^n is a proportionality constant, and A is the absorbance of light.

The amount of light absorbed can be measured by multiple ways:

$$T = P/P_o$$

$$\%T = 100T.$$

And the absorbance (A) can be calculated as:

$$A = \log_{10}\left(\frac{P_o}{P}\right)$$

$$A = \log_{10}\left(\frac{1}{T}\right)$$

$$A = \log_{10}\left(\frac{100}{\%T}\right)$$

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

The last equation ($A = 2 - \log_{10} \%T$) allows to calculate the absorbance of light from % transmittance data.

3.10.2 Lambert's Law

The rate of decrease in the intensity of incident light with the thickness of the medium is directly proportional to the intensity of incident light. It can also be stated that the intensity of emitted light is decreased exponentially as the thickness of absorbing medium is increased. It is expressed as:

$$I = Ie^{-k2l}.$$

3.10.3 Beer–Lambert Law

Beer–Lambert law (or Lambert–Beer law) describes a linear relationship between the absorbance of light and the concentration of absorbing species. The Beer–Lambert law is written as:

$$I = I_o e^{-kcl}.$$

This law is derived by combining the Beer's law and Lambert's law that associates the light absorption with the properties of sample across which the light travels.

3.10.3.1 Deviations from Beer–Lambert Law

Ideally, Beer's law is applicable only with truly monochromatic radiation. According to the Beer's law, a linear calibration curve for the absorbance of monochromatic light versus the analyte concentration in a series of standard solutions must be a straight line with an intercept of 0, but practically, calibration curve is not found to be linear (Fig. 3.11).

If the incident radiation consists of just two wavelengths λ' and λ'', with powers P'_0 and P''_0, considering that $A = -\log(P/P_0)$, then the power of the radiation to come out from (P) the cell for each wavelength would be:

$$P' = P'_0 10^{-\varepsilon' bC}$$

$$P'' = P''_0 10^{-\varepsilon'' bC}$$

where ε' and ε'' are the molar absorptivities of each wavelength. Therefore, the measured absorbance A_m may be

$$A_m = -\log\left(\frac{P' + P''}{P'_0 + P''_0}\right) = \log\left(\frac{P'_0 + P''_0}{P'_0 10^{-\varepsilon' bC} + P''_0 10^{-\varepsilon'' bC}}\right).$$

The last equation indicates a non-linear relation between A_m and C. The proportionality between A_m and C is restored only if $\varepsilon' = \varepsilon''$. The same situation

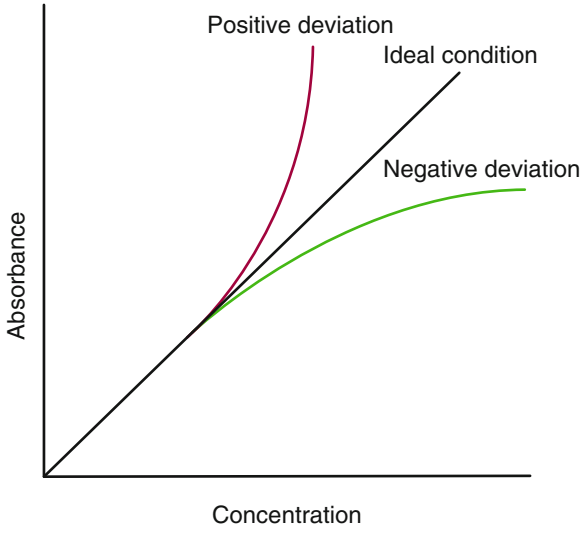


Fig. 3.11 Schematic representation of deviations from Beer's law

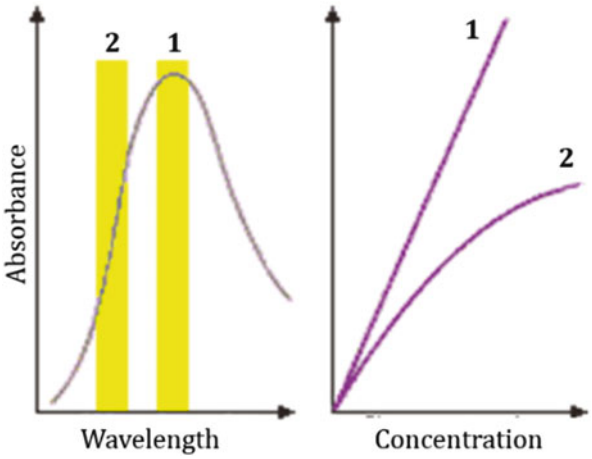


Fig. 3.12 Schematic representation of absorbance versus wavelength (left) and concentration (right)

occurs when a radiation consists of many wavelengths. The situation is illustrated in Fig. 3.12.

When a polychromatic radiation beam is used, its preferable position (in terms of central wavelength) is on the top of a relatively wide absorption peak (position 1 in Fig. 3.12). In this case, the proportionality between absorbance (A) and concentration (C) is maintained, because the molar absorptivities are practically the same for all wavelengths. On the other hand, marked deviations are expected when the band is positioned in spectral regions such as the sides of absorption peaks (position 2 in Fig. 3.12), where a wide range of molar absorptivity values is expected. It has been found that deviations from Beer's law are insignificant for the measurement of absorbance at the maximum of narrow peaks, if the effective bandwidth of the incident beam is less than 1/10 of the width of the absorption peak at half height.

3.10.3.2 Limitations from Beer–Lambert Law

Beer–Lambert law shows a direct correlation between the absorbance (A) of given analyte to the concentration (c) and path length (b) of the sample. This relationship is linear but, under certain conditions, this relationship breaks down and gives a non-linear relationship. The most important deviations from Beer–Lambert law may be categorized into the following three categories:

Real Deviations

Beer and Lambert laws describe the absorption characteristics of solutions that have relatively low concentrations (<10 mM) of solute and/or analyte dissolved in it. When analyte concentration in a given solution is higher (>10 mM), then the analyte begins to behave differently owing to the interactions with surrounding solvent molecules or other solute molecules present in solution and hydrogen bonding also plays a significant role in this regard.

Chemical Deviations

This type of deviation is observed owing to the presence of particular chemical species in sample which is being analyzed. Various factors are involved in chemical deviation notably association, dissociation, polymerization, complex formation, and interaction of analyte with solvent to make a product having different absorption properties. Followings are the examples of chemical deviations of Beer–Lambert law:

1. Phenomenon of resonance transformation occurs in phenol red. Its medium changes from acidic form (yellow) to the basic form (red) (Fig. 3.13). Due to this resonance, electron distribution of the bonds in phenol molecule changes as the pH of the solvent in which it is dissolved changes from acidic medium to the basic medium. UV-Visible spectroscopy is an electron-related phenomenon, and absorption spectrum of the sample changes with the change in pH of the solvent. Acidic and basic forms of phenol red along with their UV spectra at different pH exhibit the chemical deviations of Beer–Lambert law in UV-Visible spectroscopy.

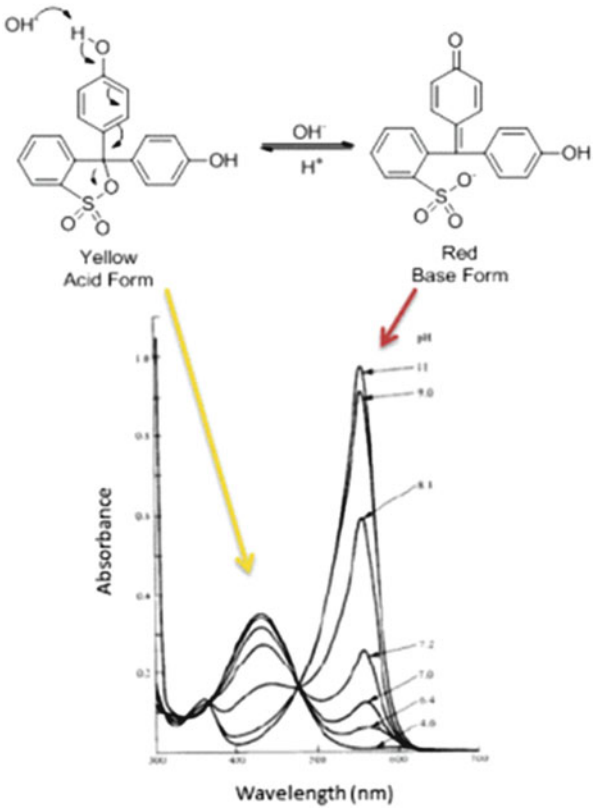


Fig. 3.13 Effect of pH on phenol red along with their UV spectra demonstrates chemical deviations of Beer–Lambert law in UV-Visible spectroscopy

2. Concentrated solution of benzoic acid in sample has lower pH and contains higher proportion of unionized form than that of dilute solution. The absorption wavelength of unionized benzoic acid is 273 nm, whereas for ionized form of benzoic acid, the absorption wavelength is 268 nm. The increased wavelength of unionized form of benzoic acid indicates that increasing the concentration of benzoic acid increases the wavelength (273 nm) with positive deviation from Beer's law. Whereas, the lower absorption of wavelength (268 nm) at low concentration of benzoic acid gives negative deviation from Beer's law.
3. Methylene blue at the concentration of 10^5 M exists as monomer with λ_{max} of 660 nm, but at concentration above 10^4 M exists as dimer with λ_{max} of 600 nm.
4. In unbuffered solution of potassium dichromate, the dissociation of dichromate ions is observed by lowering the pH.
5. Insufficient time for the completion of reaction (incomplete reaction) also produces deviation from Beer's law. For example, determination of iron using thioglycolic acid before completion of reaction.

Instrumental Deviations

These deviations may occur while measuring the absorbance of analyte. Experimental measurements are usually made in terms of transmittance (T), which is defined as:

$$T = I/I_o$$

where I_o denotes initial intensity of light and I is the intensity of light when it passes through the sample. The relationship between T and A can also be represented as follows:

$$A = -\log\ T = -\log\ (I/I_o).$$

Modern light absorption devices generally display the data either as absorbance, transmittance, or %-transmittance. Beer's law may be used in order to measure the unknown concentration of an analyte by measuring the amount of light being absorbed by the sample (Fig. 3.14). When the absorptivity coefficient is not known, the unknown concentration of analyte may be estimated by using a working curve of absorbance versus concentration derived from standards.

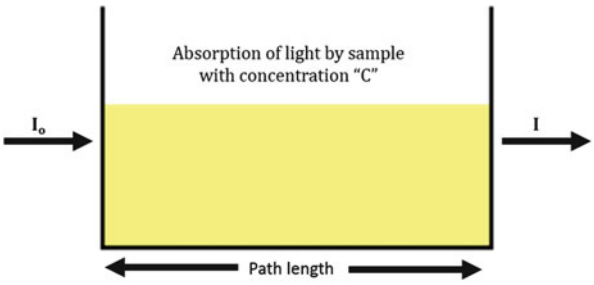


Fig. 3.14 Schematic representation of absorption of light by sample

3.10.3.3 Due to Polychromatic Radiation

Beer–Lambert law is strictly followed when a source of monochromatic light exists but practically, grafting unit (monochromator) or filter is used to create a monochromatic beam of light from the source of polychromatic light. Deviations from Beer–Lambert law are minimal, when the molar absorptivity of analyte remains essentially constant at selected wavelength on spectrometer. If the molar absorptivity of analyte changes at selected wavelength, then the absorbance of analyte does not follow Beer–Lambert law. It is observed (Fig. 3.15) that the deviations in absorbance over the wavelengths are minimal when the wavelength observed is at the λ_{max}. Due to this reason absorption measurements are taken at wavelengths.

Figure 3.15 shows a difference in deviations in absorbance when values are obtained at λ_{max} of absorbance (band A) vs. other wavelengths of absorbance (band B). Figure 3.15b shows the deviations in Beer–Lambert law due to the observations made at wavelengths other than λ_{max}.

3.10.3.4 Due to the Presence of Scattered Radiation

Scattered radiation refers to radiation from the device that is outside the nominal selected wavelength band. Generally scattered radiation wavelength varies from the

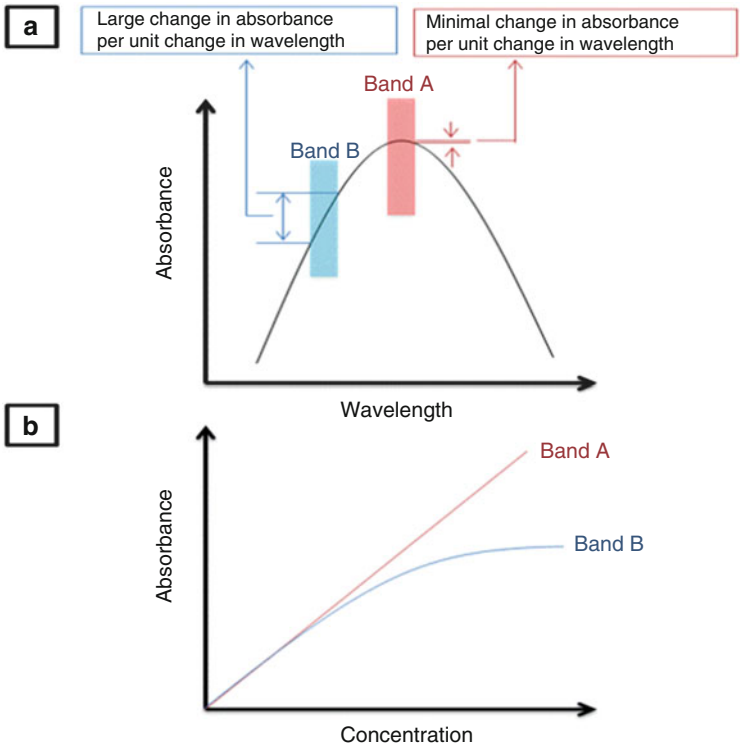


Fig. 3.15 Deviation from Beer–Lambert law due to polychromatic radiation

selected wavelength band. It has been observed that radiations from a monochromator are often contaminated with small quantity of scattered radiation. Usually, scattered radiation is produced due to the reflection and/or scattering of light by the surface of grating, mirrors, filters, lenses, and windows. When the analyte absorbs at the wavelength of the scattered radiation, a deviation from Beer–Lambert law is observed comparable to the deviation due to the polychromatic radiation.

3.10.3.5 Due to Mismatched Cuvettes

Beer–Lambert law cannot be followed when the cuvettes containing the analyte and the blank solutions have variable path lengths, or unequal optical characteristics. In such cases when absorbance versus concentration is plotted, the curve will have an intercept k and the equation will be defined as:

$$A = \varepsilon bc + k.$$

3.10.3.6 Theoretical Limitations

Beer's law is applicable only if the concentration of analyte is low. At higher concentrations, the individual particles of analyte no longer behave independently with one another and do not follow this law. The resulting interaction between the particles of analyte may change the value of a or ε. The values of a or ε depend on sample's refractive index, whereas the refractive index varies with the analyte's concentration.

3.11 Terms Used in UV-VIS Spectroscopy

In UV-VIS spectrophotometry, two main terms, namely chromophore and auxochrome, are used. The detailed descriptions of these two terms are as follows:

3.11.1 Chromophore

Chromophore is derived from the Greek word "Chromophorus" which mean the color carrier and it can be defined as "the part of a molecule that is covalently bonded with unsaturated group responsible for imparting the color after absorbing the light in UV or VIS region" is called as chromosphere, or it can also be defined as "the functional group containing multiple bonds capable of absorbing radiations above 200 nm." It can also be defined as "any structural feature that is present in the molecule and responsible to absorb EMR and hence impart color to the compound." For example, in nitro compounds, the yellow color is produced due to presence of NO_2 group and hence NO_2 is considered as chromophore. Typical examples of chromophore are NO_2, N=O, C=C, C=N, C≡N, C=O, C=S, etc.

White light is composed of various colors (radiations). When a specific compound is placed in the path of white light, a specific color is produced in human eye

Table 3.1 Various regions of visible spectrum and their corresponding colors

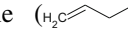
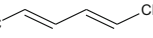
Region	Complementary color	Wavelength (nm)
Violet	Yellow-green	400–435
Blue	Yellow	435–480
Green-blue	Orange	480–490
Blue-green	Red	490–500
Green	Purple	500–560
Yellow-green	Violet	560–580
Yellow	Blue	580–595
Orange	Green-blue	595–650
Red	Blue-green	650–800

because it absorbs some specific color from the white light while transmitting the remaining colors. The precise color of the compound that it reflects depends on the wavelength it absorbs from the white light. For instance, if a compound absorbs blue light (435–480 nm) from the white light, it will transmit the yellow light; then compound will produce the yellow light to the human eye. The transmitted or reflected color from the compound is known as the complementary color of the absorbed color. Table 3.1 contains the complementary color of the white light which can be produced from each region.

All the compounds that contain chromophore in their molecules generally contain π electrons and many of them also contain non-bonding (n) electrons. Those functional groups that contain both n and π electrons undergo three types of electronic transitions, i.e., $n \rightarrow \pi^*$, $\pi \rightarrow \pi^*$, and $n \rightarrow \sigma^*$, in addition to $\sigma \rightarrow \sigma^*$ electronic transitions.

Saturated hydrocarbons only contain σ electrons and hence $\sigma \rightarrow \sigma^*$ electronic transitions are involved in these compounds which require energy that is only available in vacuum UV region. Therefore, saturated hydrocarbons do not show absorption in UV-VIS region (200–800 nm) and thus can be used as solvent for the spectral analysis throughout this region. Similarly, saturated compounds that possess heteroatoms, such as halogens, nitrogen, and oxygen, contain non-bonding (n) electrons in addition to σ electrons. That is why, in addition to $\sigma \rightarrow \sigma^*$ electronic transition, $n \rightarrow \sigma^*$ electronic transition is involved and majority of such types of compounds that involve $\sigma \rightarrow \sigma^*$ electronic transition do not show absorption in UV-VIS region and hence are commonly used as solvent for spectral analysis of analyte.

Following points should also be considered to interpret UV-VIS spectrum:

1. Non-conjugated alkenes have the absorbance at less than 200 nm of wavelength. Therefore, they are inaccessible to UV spectrophotometer. For example, if double bonds are conjugated in a compound, λ_{max} is shifted towards longer wavelength. 1,5-hexadiene () has $\lambda_{max} = 178$ nm and 2,4-hexadiene () has $\lambda_{max} = 227$ nm.

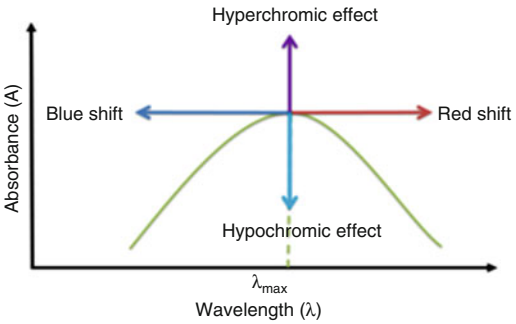
2. Non-conjugated compound with carbonyl group gives a weak absorption band in the 200–300 nm region. For example, acetone () which has $\lambda_{max} = 279$ nm and that cyclohexane () has $\lambda_{max} = 291$ nm.
3. Conjugation of C=C and carbonyl group shifts the λ_{max} of both groups to a longer wavelength. For example, ethylene ($H_2C{=}CH_2$) has $\lambda_{max} = 171$ nm, acetone () has $\lambda_{max} = 279$ nm, and crotonaldehyde () has $\lambda_{max} = 290$ nm.

3.11.2 Auxochrome

The wavelength of absorption maxima for specific molecule or compound not only depends on the nature of the functional group present in the molecule, but also on the nature of chromophore. There are certain functional groups, such as –SH, $–NH_2$, –OH, and halogens, which are not chromophore themselves (do not show absorption at wavelength higher than 200 nm), once they are attached to a chromophore, they enhance the absorption of chromophore to shift towards a longer wavelength, accompanied by an increased intensity. Such functional groups are known as auxochromes. Auxochromes when attached to a particular chromophore, they alter the ability of a chromophore to absorb light and alter the intensity of light absorption. Auxochrome in fact is a color enhancing group that contains non-bonding (n) electrons that do not absorb EMR themselves in near UV region, but when they attached to a chromophore, alter the wavelength and intensity of absorption of that chromophore. Therefore, they can be defined as "any group whose presence bring about the shift of absorption band towards a longer wavelength." For example, benzene () shows λ_{max} at 255 nm, but if any group (e.g., –OH) when attached to benzene, it increases the λ_{max} such as phenol (), it has λ_{max} at 270 nm. In phenol, –OH group is an auxochrome. Similarly, when $–NH_2$ group is attached to benzene ring, then it increases the λ_{max} towards longer wavelength (aniline () has λ_{max} at 280 nm) as compared to that of –OH group which indicates that $–NH_2$ group is more powerful than the –OH group.

3.12 Absorption and Intensity Shifts in UV-VIS Spectroscopy

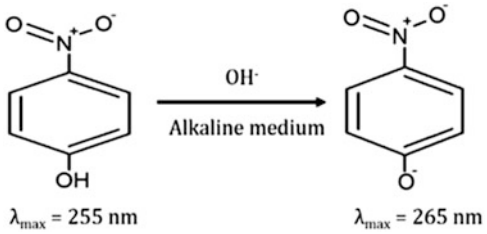
The subsituent group when attached on a basic structure of chromophore and replaces hydrogen, cause the change of the intensity and position of absorption band of the chromophore. The subsituent groups do not show the absorption in UV-radiation themselves. But due to the presence of substituent group, absorption of the principal chromophore is modified. Subsituents (possibly the auxochromes) may either increase or decrease the intensity of absorption, and the possibily the



Descriptive term	Nature of the shift
Bathochromic shift (Red shift)	Towards longer wavelength
Hypsochromic shift (Blue shift)	Towards shorter wavelength
Hyperchromic effect	Towards higher absorbance
Hypochromic effect	Towards lower absorbance

Fig. 3.16 Schematic representation of absorption and intensity shifts (upper part of the figure) and their relevant descriptive terms (lower part of the figure in the form of table)

Fig. 3.17 Effect of hydroxyl group on absorption maxima of p-nitrophenol



wavelengths as well (Fig. 3.16). Following are the four types of absorption and intensity shifts:

3.12.1 Bathochromic Shift (Red Shift)

If λ_{max} of the given compound shifts towards the longer wavelength, this type of shift is known as bathochromic shift or red shift. Bathochromatic shift is produced due to the presence of an auxochrome in compound or by changing the pH of the medium in which compound is present. For example, auxochrome groups such as –OH, $–OCH_3$ when attached on a compound, increase the absorption of compound at longer wavelength. p-nitrophenol shows bathohromic shift in alkaline medium (Fig. 3.17) due to the negatively charged oxygen atom that delocalizes more effectively as compared to unshared pair of electron.

3.12.2 Hypsochromic Shift (Blue Shift)

If λ_{max} of the given compound shifts towards the shorter wavelength, this type of shift is known as hypsochromic shift or blue shift. Hypsochromic shift is produced due to the presence of functional group that causes the removal of conjugation or with pH change of the medium in which compound is present. For example, aniline shows hypsochromic shift in acidic medium, because it loses conjugation (Fig. 3.18).

3.12.3 Hyperchromic Shift

If absorption intensity (ε) of the given compound increases, this type of shift is called as hyperchromic shift. It may occur due to introduction of an auxochrome which ultimately results in the increase in intensity of coumpound. For example, when methyl group (auxochrome) is introduced in the structure of pyridine, it increases the intensity of pyridine from 2750 to 3560 (Fig. 3.19).

Hyperchromic effect may also occur due to the change of solvent. For example, phenol shows bathochromic shift along with hyperchromic effect in the presence of alkaline media (Fig. 3.20).

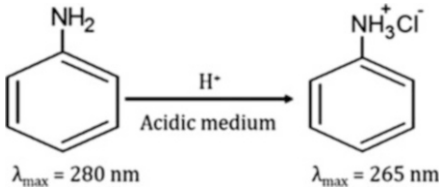


Fig. 3.18 Effect of acidic medium on absorption maxima of aniline

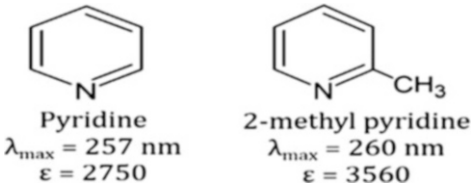


Fig. 3.19 Effect of auxochrome on intensity of pyridine

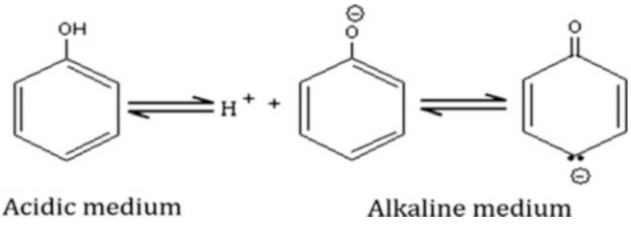


Fig. 3.20 Effect of medium on bathochromic shift along with hyperchromic effect

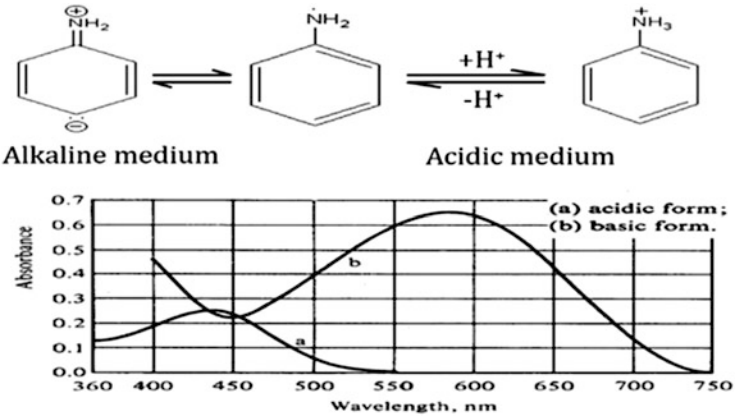
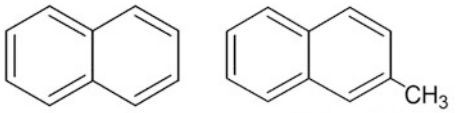


Fig. 3.21 Acidic medium shows hypsochromic shift and hypochromic effect in aniline

Fig. 3.22 Effect of auxochrome on absorption intensity of naphthalene



Naphthalene
ε = 19000

2-methyl naphthalene
ε = 10250

3.12.4 Hypochromic Shift

If absorption intensity (ε) of the given compound decreases, this type of shift is called as hypochromic shift. For example, aniline shows hypsochromic and hypochromic shift in acidic medium. For example, analine shows hypochromic effect and hypsochromic shift in acidic medium (Fig. 3.21).

It may occur due to the introduction of an auxochrome which ultimately results in the decrease in intensity of coumpound. For example, naphthalene shows absorption intensity (ε) at 19,000, but when methyl group (auxochrome) is introduced in the structure of naphthalene, it decreases the intensity of 2-methyl naphthalene from 19,000 to 10,250 (Fig. 3.22).

3.13 Applications

UV-VIS spectroscopy is regularly used in different fields of science for the qualitative and/or quantitative analysis of different types of analytes. The analysis is usually carried out when analyte is in solution form but the analyte presents even in gas or solid, may also be analyzed using UV-VIS spectroscopy. Followings are the important applications of UV-VIS spectroscopy:

3.13.1 Determination of Molecular Weight

Molecular weights of the organic compounds may be determined with the help of UV-VIS spectroscopy. This method is based upon the formation of a derivative.

3.13.2 Detection of Impurities

UV-VIS spectrophotometric analysis is considered as the best method for detection of impurities that may be present in organic molecules. Additional peaks observed in the spectra of pure compound indicate the presence of impurities in pure material (Fig. 3.23).

3.13.3 Quantitative Analysis

UV-VIS spectroscopy may be utilized for the quantitative analysis of compounds. This analysis is based on the Beer–Lambert law. Various drugs either in the form of raw material or in the form of final dosage form may be analyzed by making a solution of given drug in a suitable solvent and measuring the absorbance at particular wavelength. For instance, diazepam tablets may be analyzed by measuring the absorbance of diazepam in 0.5% sulfuric acid in methanol at 284 nm wavelength. Quantitative analysis of the drug can be done by using reference standard and/or multiple standard methods and single compound analysis by using separation technique after extraction and/or after chromatographic separation.

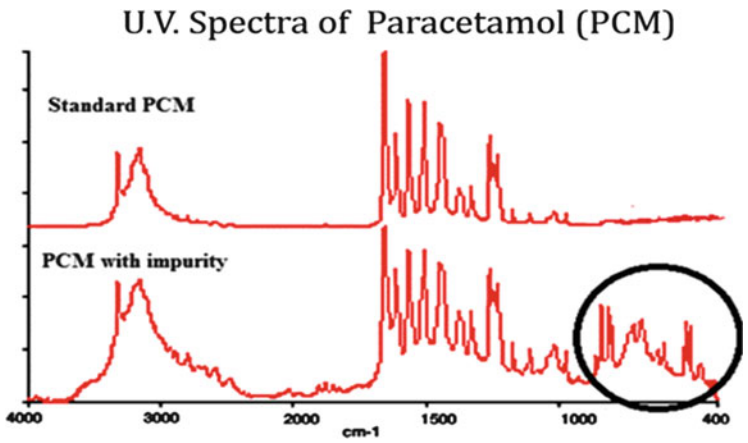


Fig. 3.23 Schematic representation of UV-VIS spectra of standard PCM and/or with impurity

3.13.4 Qualitative Analysis of Pharmaceuticals

UV-VIS absorption spectroscopy can be used to characterize those compounds which absorb UV and/or visible radiation. Identification is done by comparing the absorption spectra of unknown compound and/or analyte with the spectra of known compounds. A record of UV-VIS absorption curves can be found in certain reference books.

3.13.5 Detection of Functional Group

It is possible to detect the presence of certain functional group in compound with the help of UV–VIS spectroscopy. Absence of a band at particular wavelength indicates the evidence for absence of particular group in compound (Fig. 3.24).

3.13.6 Chemical Kinetics

UV-Visible spectroscopy can be used to study the chemical kinetics of the rate of chemical reactions. In order to determine the kinetics of reaction, the change in the concentration of either a reactant or product with respect to time is measured. As absorbance is directly proportional to the concentration of analyte, UV-VIS spectrophotometry can be used to follow the course of a reaction. The method is based upon the fact that one of the reactants of product exhibiting a suitable absorption in UV region is not overlapped by absorption spectra of other species that may be present in sample. This method can be employed to study such rates which must be relatively slow (half-lives of the order of a minute).

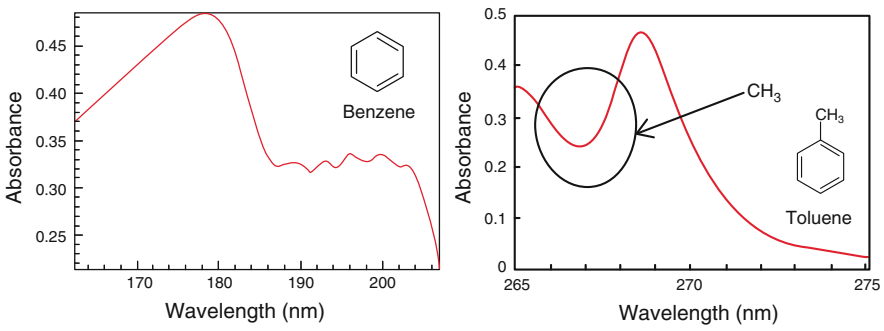


Fig. 3.24 Schematic representation for detection of functional group using UV-VIS spectroscopy

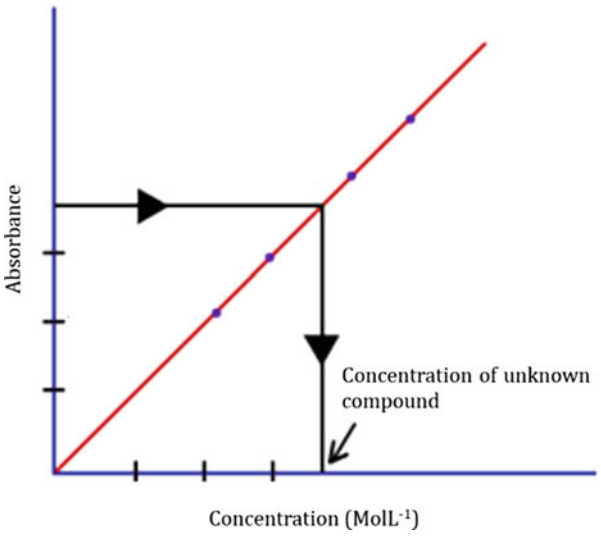


Fig. 3.25 Schematic representation for determination of unknown concentration of a compound using UV-VIS spectroscopy

3.13.7 Determination of Unknown Concentration

Unknown concentration of any compound can also be measured using UV-VIS spectroscopy (Fig. 3.25). It involves the following steps:

1. First of all, prepare the samples.
2. Then prepare series of standard solutions having known concentrations.
3. Set spectrophotometer to the wavelength at which sample shows maximum absorption of light.
4. Draw the standard plot.
5. Finally, measure the absorption of unknown analyte and determine its concentration with the help of standard plot.

3.13.8 Determination of Extent of Conjugation

It is helpful to determine the relationship between various groups mainly with respect to conjugation that usually can occur between the

- Two or more carbon–carbon double or triple bonds, e.g., in aromatic compounds.
- Double bonds between carbon and oxygen.
- Double bonds present in various other organic compounds.

It may reveal the existence of an aromatic ring and total number and locations of various substituents that are attached to the carbons of conjugated system.

3.13.9 Structural Elucidation of Organic Compounds

From the location and combination of peaks, structural elucidation of organic compounds can also be done using UV-VIS spectroscopy. It indicates the degree of unsaturation and existence of heteroatoms in organic compounds.

3.13.10 As HPLC Detector

UV-VIS spectrophotometer can also be utilized as a detector in combination with HPLC. The presence of an analyte gives a response which can be assumed to be proportional to the concentration.

3.14 Advantages

1. Analysis is quick.
2. Sample analysis is easy.
3. Absorption spectrum provides valuable information regarding the presence of analyte in sample.

3.15 Disadvantages

1. Lack of sensitivity and selectivity.
2. Limited to UV/VIS absorbing compounds.
3. Need spectrophotometer capable of reading in the UV-VIS region.
4. Samples should be in solution form.
5. Mixture of substances poses difficulty to analyze and requires prior separation.
6. Interferences from sample mixture make the measurement difficult.
7. Samples should be in solution. Mixture of substances poses difficulty to analyze and requires prior separation.
8. Interference from the sample's matrix makes the measurement difficult.

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Infrared Spectroscopy

4

Abstract

Infrared spectroscopy deals with the interaction of infrared (IR) region of electromagnetic spectrum with that of analyte present in sample. The absorption of electromagnetic radiation (EMR) in IR region is based on resonant frequency, i.e., absorption happens when natural frequency of vibration of bonds and groups matches with applied frequency. In this chapter, we have comprehensively described the various types of vibration of bonds or groups. There are multiple components associated with IR spectroscopy that are needed for its proper functioning. IR spectroscopy is useful for the analysis of sample existing in any form either solid, liquid, or gas. However, different sampling techniques are used for the preparation of sample to be analyzed by IR spectroscopy. A comparison of different types of IR spectroscopy is also given in this chapter. At the end, various applications of this spectroscopy have been described along with advantages and disadvantages.

Keywords

Regions of IR · Modes of molecular vibrations · Sampling techniques · Types of IR spectroscopy · Interpretations of IR spectra

4.1 Introduction

Infrared spectroscopy is used to determine the absorbance of given sample within the infrared (IR) region of the electromagnetic radiations (EMR). IR radiation exists between the microwave and visible regions of EMR. Wavelength of IR radiation is longer than visible but smaller than microwaves. However, frequency of IR radiation is lower than visible but greater than microwaves. IR is a kind of EMR which has wavelength region longer than visible light, but shorter than radio wave. Different regions of EMR have been shown in Fig. 4.1.

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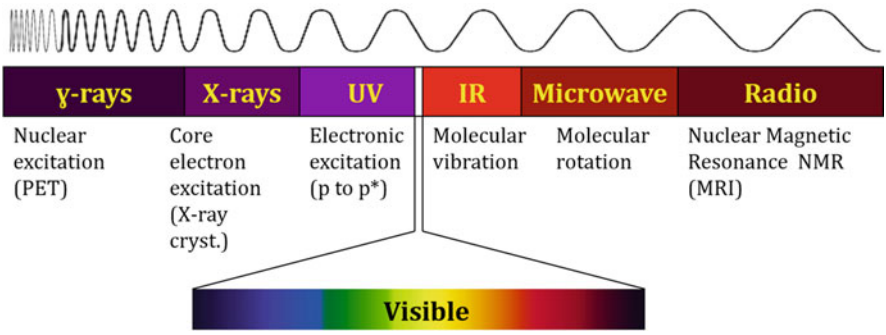


Fig. 4.1 Schematic representation of regions of electromagnetic radiations

Table 4.1 Regions of IR and their ranges

IR regions	Range
Near IR region	0.8–2.5 μm (12,500–4000 cm^{-1})
Mid IR region	2.5–15 μm (4000–667 cm^{-1})
Far IR region	15–200 μm (667–100 cm^{-1})

4.2 Regions of IR

There are three types of IR regions that have been described in Table 4.1. The unit commonly used is wavenumbers (cm^{-1}), i.e., 4000–400 cm^{-1}. Wavenumber is proportional to energy (E) and frequency (v), but reciprocal to wavelength.

4.3 Principle

Molecules consist of atoms that are linked by different chemical bonds. The movement of atoms and their associated chemical bonds resembles with spring and balls system. This characteristic movement is known as natural frequency of vibration. Applied IR frequency is equal to the natural frequency of vibration. Owing to vibrational motion, there is a change in dipole moment of the molecule. Covalent bond oscillation results in oscillation of dipole of the molecule producing an electromagnetic (EM) field. The more dipole moment changes because of the vibrational motion, the more intense the electromagnetic field is produced. Basically, IR spectroscopy is the measurement of the reflection, absorption, and emission of IR spectrum.

4.4 Modes of Molecular Vibrations

Any slight change in the shape of molecule via bending of bonds, stretching of bonds, or internal rotation around a single bond is known as molecular vibration (Fig. 4.2). When the waves of EMR interact with matter, they change the appearance of bending and/or stretching vibrations. Based on the types of vibrations that appear in the form of bands in spectra, molecular vibrations are classified into the following types.

4.4.1 Stretching Vibration

The vibration along the line of bond is known as stretching vibrations. These vibrations occur in radial direction and change bond length either by increasing the bond length or decreasing the bond length. These vibrations also involve the change in interatomic distance along the axis of the bond between the two atoms. Such types of vibrations require higher energy, i.e., 4000–1250 cm^{-1} and appear at shorter wavelength. Stretching vibrations have the following two types:

4.4.1.1 Symmetrical Stretching Vibration

In this kind of stretching, the interatomic distance between the two bonds increases or decreases simultaneously (Fig. 4.3).

4.4.1.2 Asymmetrical Stretching Vibration

In this kind of stretching, the length of one bond is increased and the length of another bond is decreased. The interatomic distance between the two atoms is alternate and/or opposite to each other (Fig. 4.4).

4.4.2 Bending Vibrations

These types of molecular vibrations are either latitudinal or longitudinal in direction that results in the change of angle between the two adjacent bonds. Latitudinal

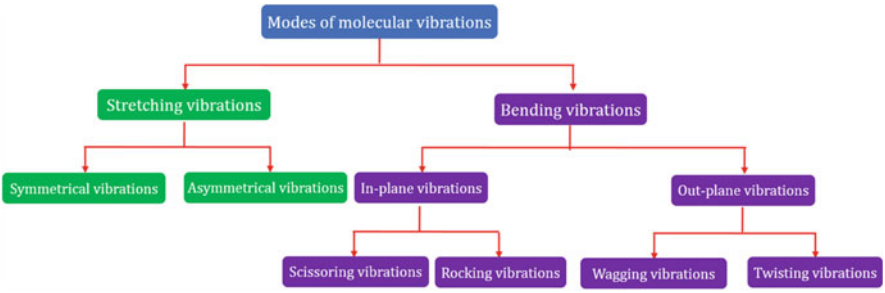


Fig. 4.2 Schematic representation of modes of molecular vibrations

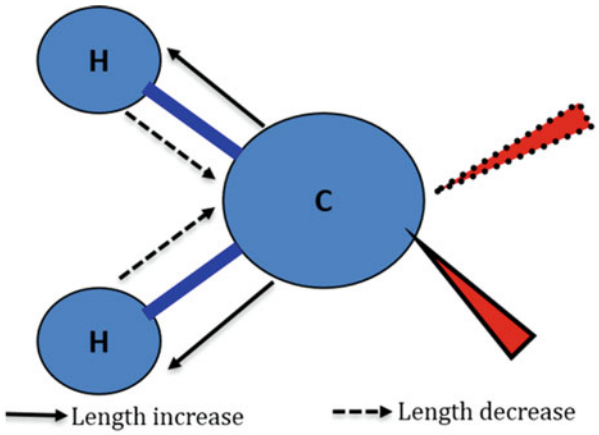


Fig. 4.3 Schematic representation of stretching vibration

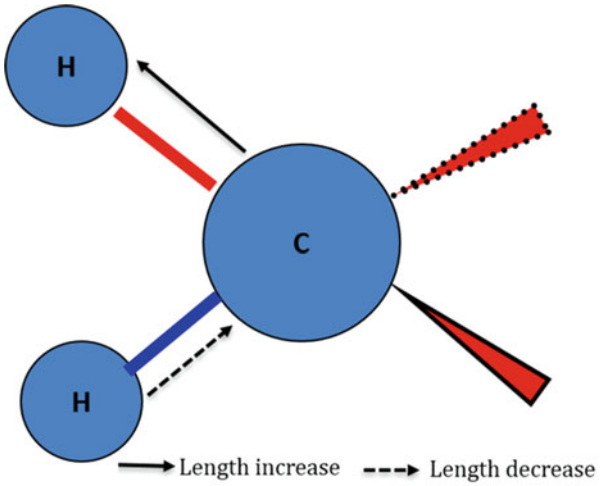


Fig. 4.4 Schematic representation of stretching vibration

bending vibrations are known as in-plane bending vibrations, whereas the longitudinal bending vibrations are known as out-plane bending vibrations. These vibrations require less energy so easily happens at higher wavelength.

4.4.2.1 In-Plane Bending Vibrations

In-plane bending vibrations involve a change in bond angle and they occur in the same plane. These are of the following two types:

Scissoring Vibration

Scissoring vibration happens when two atoms approach to each other. The bond angles are decreased in this type of vibration (Fig. 4.5).

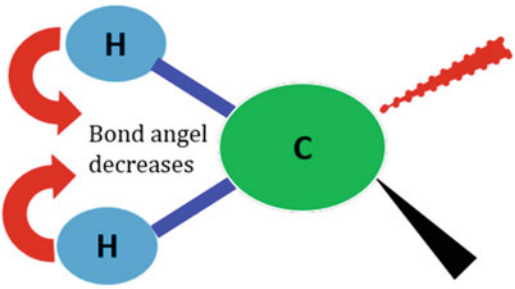


Fig. 4.5 Schematic representation of scissoring vibration

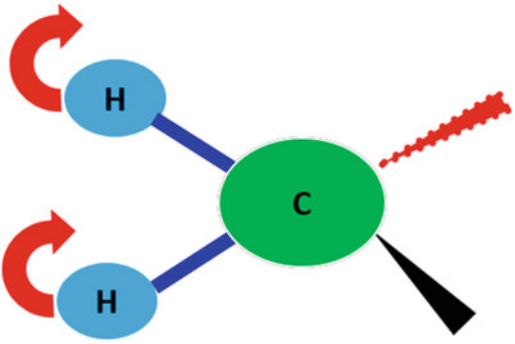


Fig. 4.6 Schematic representation of rocking vibration

Rocking Vibration

Rocking vibration happens when movement of atoms occurs towards the same direction without any change in the angle of bond (Fig. 4.6).

4.4.2.2 Out-Plane Bending Vibrations

As the name indicates, out-plane bending vibrations take place away from the plane. These are of the following two types:

Wagging Vibration

Wagging vibration happens when two atoms move towards the same side of the plane. They move either up or down of the plane (Fig. 4.7).

Twisting Vibration

Twisting vibration happens when two atoms move to opposite sides of the plane. One atom moves up from the plane and second moves down from the plane (Fig. 4.8).

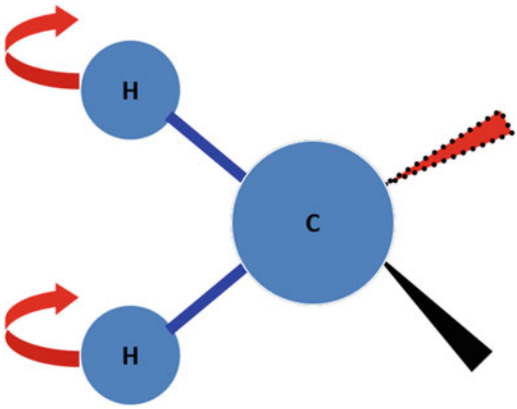


Fig. 4.7 Schematic representation of wagging vibration

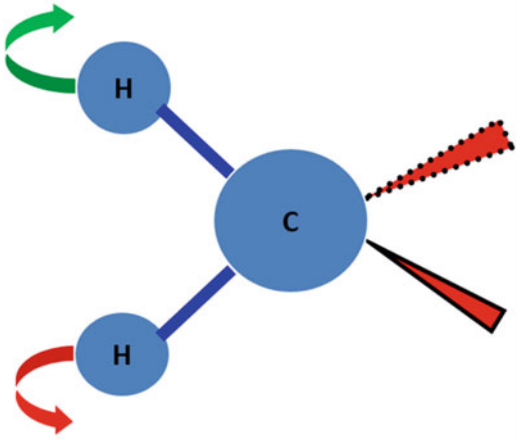


Fig. 4.8 Schematic representation of twisting vibration

4.5 Components of IR Spectrophotometer

The following are the components of IR spectrophotometer:

4.5.1 Radiation Source

Radiation source is an important component of IR spectrophotometer. It continuously emits IR radiation having sufficient intensity. Radiation source should have the following properties:

1. Intensity of radiation should be continuous over the λ range and covers a wide λ range.

2. Intensity of radiation should be constant over long periods of time.
3. Source should have the normal operating temperatures, i.e., between 1100 and 1500 K.
4. Source should have maximum intensity between 4000 and 400 cm^{-1}.
5. Sources should be enclosed in an insulator to reduce noise.

The important examples of modern sources for radiations include furnace ignitors, diesel engine heaters. The following are the important sources of IR radiation:

4.5.1.1 Mid-IR Sources

The sources for mid-IR radiation have the following types:

1. *Nernst glowers*: It is made up of zirconium oxide, cerium oxide, and/or thorium oxide. It contains heated ceramic rods with cylindrical bar. It can be heated electrically up to 1500 °C and emits radiations between 0.4 and 20 μm. As the temperature increases, resistance decreases proportionally. If the care is not taken, overheating may cause the burning of overall apparatus.
2. *Globar*: It contains bar that is made of silicon carbide which can be heated electrically to emit the continuous IR radiations. It is more sensitive than the Nernst glower.
3. *Heated wire coils*: It can also be heated electrically up to ~1100 °C. Its shape is similar to the incandescent light bulb filament. Nichrome wire is commonly used but sometimes, rhodium is also used. Due to the softness and flexibility in wire, it may easily fracture and burn out.

4.5.1.2 Near-IR Sources

One of the most important examples of sources for near-IR radiation is quartz halogen lamp. It contains tungsten wire filament in which iodine vapors are sealed in a quartz bulb. Upon heating, the tungsten is evaporated and then redeposited on the filament. This phenomenon of tungsten redeposition on filament increases the stability. The output range is between 25,000 and 2000 cm^{-1}.

4.5.1.3 Far-IR Sources

The most important example of far-IR sources for radiation is high pressure mercury discharge lamp which contains inert gas and two electrodes. It is made of quartz bulb that contains elemental mercury.

4.5.1.4 Laser Sources for IR Radiation

It has the following two types:

1. Gas-phase (tunable CO_2) laser.
2. Solid-state laser.

4.5.2 Sample Cell

It holds the sample and allows the monochromatic light to pass through it for the measurement of IR spectra. The samples to be analyzed may be in solid, liquid, or gaseous forms. It is usually made up of alkali halides, e.g., NaCl or KBr. These halides are soluble in water; therefore, aqueous solvents cannot be used for analysis because these solvents dissolve the sample cell if it is composed of alkali halides. Only organic solvents such as carbon disulfide (CS_2) and carbon tetrachloride (CCl_4) are used for analytical purpose.

4.5.3 Monochromator

Monochromator is used to split the polychromatic radiation into component wavelengths either by using the prisms (metal halide or NaCl prism) or grating or both. Quality of mirrors and slit width determine the resolution of IR spectrum. Rock salt prism that is made up of metal with polished front surface is usually used in the range of 650–4000 cm^{-1}.

4.5.4 Detectors

Detector is a device that receives the light signals from the sample cell and converts it into electrical signals.

4.5.4.1 Types of Detectors

Depending upon the characteristics of the material used in the composition of the detector and nature of the analyte whose spectrum is to be determined, there are two types of the detectors which are as follows:

Thermal Detectors

They consist of a tiny active element used for the detection. Insulation of that element or decreased size of that element increases the detector response. Such detectors are widely used because of linearity in response but they have some demerits including slow processing time and less sensitivity.

1. *Bolometers*: These are sensitive to electrical resistance. The older ones were made up of platinum wire (resistance change = 0.4% per °C). Modern ones are made of silicon that is placed in a Wheatstone bridge. Their diameter is few micrometers and their response time is very fast.
2. *Thermocouples*: These detectors are made up of two wires from different metals, welded together at both ends, e.g., bismuth and antimony. One welded joint known as hot junction is exposed to IR radiation. The other joint known as cold junction is kept at constant temperature. Hot junction becomes hotter than the

cold junction. Potential difference between the hot and cold junction is a function of IR radiation. It has slow response time and cannot be used for FTIR spectrophotometry.

3. *Thermistors*: These detectors contain fused mixture of various metal oxides. Owing to increase in temperature, there is a decrease in resistance in these detectors which is a function of IR radiation. The approximate resistance change is about 5% per °C but the response time is very slow.
4. *Pyroelectric devices*: These are made of ferroelectric materials, dielectric materials, or semiconductors. A thin crystal of a material is placed between the two electrodes. Any change in the temperature changes the polarization of the material which is a function of IR radiation exposed. The electrodes measure the change in polarization in response to the change in temperature. They have a fast response time.

Photon-Sensitive Detectors

These are made of materials such as indium antimonide, indium gallium arsenide, lead selenide. These materials are semiconductor in nature but they are very sensitive and have very fast response time.

4.5.5 Read-Out Device

It receives the electrical signals from the detector and translates it into easy to interpret form.

4.6 Sampling Techniques for IR Spectroscopy

In IR spectroscopy, the sample used for analysis is in the form of solid, liquid, or gas. Inappropriate sample influences the overall results of analysis. Therefore, keeping in view the nature of the sample, different sampling techniques are used which are as follows:

4.6.1 Solid Samples

In case of solid samples, following techniques may either be used to prepare the solid samples for IR spectroscopy:

4.6.1.1 Mulling

In this technique, the solid sample is ground to make it powder (Fig. 4.9). Few drops of viscous liquid like Nujol are added to make thick slurry of the sample which is then pressed between salt plates to form a thin film.

Fig. 4.9 Schematic representation of solid sample preparation mulling technique

Fig. 4.10 Schematic representation of solid sample preparation pelleting technique

4.6.1.2 Pelleting

In this technique, 1 mg of the ground sample is mixed with 100 mg of dry powder of KBr. Mixture is then compressed under very high pressure and small disk with very smooth surfaces is formed that looks like a glass (Fig. 4.10).

4.6.1.3 Thin Film Formation

Molten sample is deposited on the surface of NaCl or KBr plates and sample is then allowed to dry to form a thin film on the substrate. This technique is good for qualitative identification of polymers but it cannot be used for quantitative analysis.

4.6.2 Liquid Samples

Many liquid samples are analyzed as it is. There is no need to use a specific technique to prepare the liquid samples if IR spectroscopy is to be carried out but dilution with an appropriate solvent such as CCl_4, CS_2, and CH_3Cl may be necessary. If the solvent is used for the dilution of liquid sample, then the solvent must be transparent in the region of interest. Salt plates are hygroscopic in nature and water-soluble samples should be avoided to be in contact with these plates. Therefore, special cells like BaF_2 and AgCl are used for water containing samples.

4.6.3 Gas Samples

Like the liquid samples, gas samples do not require any special technique to prepare sample for IR spectroscopy. For gaseous samples, special gas cells are used. These cells have longer path lengths generally about 10 cm (commercial ones are up to 120 cm) to compensate very low concentrations of gas samples. For quantitative analysis, both temperature and pressure are controlled.

4.7 Types of IR Spectroscopy

The following are the types of IR spectroscopy:

4.7.1 Dispersive IR Spectroscopy

It contains filter and/or grating monochromator. The main components of dispersive IR spectrometer are radiation source, monochromator, and detector. Dispersive spectrometers usually contain a double-beam design having two equivalent beams coming from the same source and pass through the reference and sample chambers as an independent beam.

4.7.2 FT-IR Spectroscopy

Fourier-transform infrared (FT-IR) spectroscopy is one of the most important types of IR spectroscopy which is used to obtain the IR spectra of high resolution over the wide range of spectrum either by the absorption or emission of the analyte which is either solid, liquid, and/or gaseous in nature. Generally, FT-IR spectrometer contains a source of radiation, interferometer, sample compartment, detector, amplifier, analog-to-digital converter, and computer. The source is used to generate radiations that pass through the sample via interferometer and reach to the detector. The amplifier amplifies the signals and converts them to digital

Table 4.2 Difference between dispersive IR spectroscopy and FT-IR spectroscopy

S. No.	Dispersive IR spectroscopy	FT-IR spectroscopy
1.	There are numerous moving parts, causing mechanical slippage	Mirror is the only part that moves during the experiment
2.	Calibration against the reference spectra is needed to measure the frequency	Use of laser provides greater frequency accuracy (up to 0.01 cm^{-1})
3.	Stray light gives spurious readings	Stray light do not affect the detector
4.	Only a small amount of IR beam is allowed to pass to increase the resolution	A much larger beam is used at all time. Data collection is comparatively easy
5.	Only radiation of a narrow frequency range falls on the detector at one time	All frequency of radiation falls on the detector simultaneously
6.	Scanning speed is slow	Scanning speed is high

signals by analog-to-digital converter. Ultimately, the signals are transferred to the computer in which FT is carried out.

The difference between the dispersive IR and FT-IR spectroscopy is described in Table 4.2:

4.7.3 Near-IR Spectroscopy

It is used for the quantitative analysis of liquid and solid samples having OH, NH, CH bonds. It is commonly used for quantitative characterization of polymers, food, proteins, and agricultural products. Pharmaceutical tablets can be analyzed nondestructively with the help of this technique. Forensic analysis of unknown wrapped powders that may be believed to be drugs and/or narcotics, are analyzed without destroying the wrappers.

4.8 Regions of IR Spectrum

In IR spectra, there are four primary regions which may help to interpret the spectra of unknown compound. These four regions of IR spectra have been described in Fig. 4.11.

4.9 Interpretations of IR Spectrum

Bending and stretching vibrations occur with a particular frequency as the charges and atoms involved are different for different bonds. A schematic representation of IR spectra has been illustrated in Fig. 4.12. In the following sub-sections, IR spectra of few compounds have been interpreted accordingly.

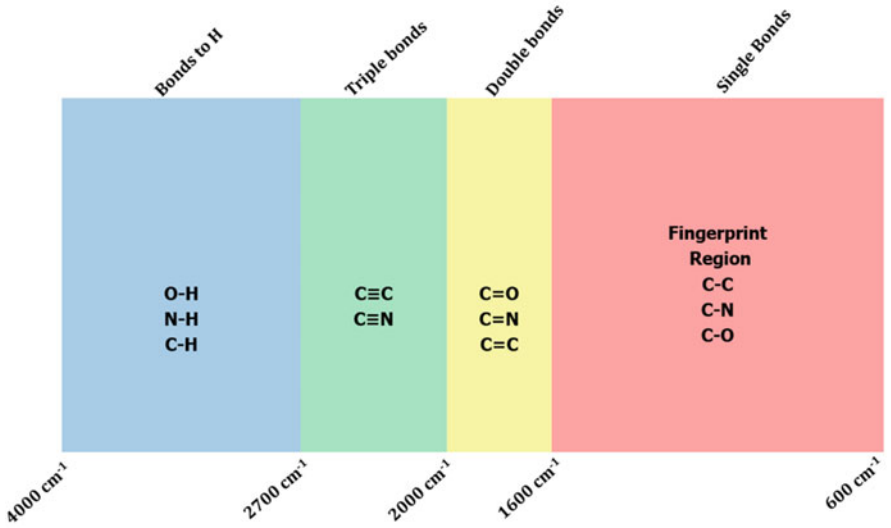


Fig. 4.11 Schematic representation of different regions of IR

4.9.1 IR Spectra of Alkanes

Consider the IR spectra of octane (Fig. 4.13). The combination of C–H and C–C bonds (e.g., octane) is as follows: C–C stretching and bending appears between 1360 and 1470 cm^{-1}, CH_2–CH_2 bond appears between 1450 and 1470 cm^{-1}, CH_2–CH_3 bond appears between 1360 and 1390 cm^{-1}, sp^3 C–H bond between 2800 and 3000 cm^{-1}.

4.9.2 IR Spectra of Alkenes

Consider the spectra of 1-octene (Fig. 4.14). Addition of the vinyl C–H and C=C bonds (e.g., 1 octene) is as follows: C=C stretching is appeared at 1620–1680 cm^{-1} which becomes weaker as the substitution increases in the structure. Vinyl C–H stretch happens at 3000–3100 cm^{-1}.

4.9.3 IR Spectra of Alkynes

Consider the spectra of 1-octyne (Fig. 4.15). Addition of C≡C and vinyl C–H bonds (e.g., 1 octyne) is as follows: C≡C stretch appears between 2100 and 2260 cm^{-1}. Strength of C≡C depends on asymmetry of bond. This bond is strongest if C≡C is present at terminal alkynes and weakest for symmetrical internal alkynes. C–H for terminal alkynes appears between 3200 and 3300 cm^{-1}.

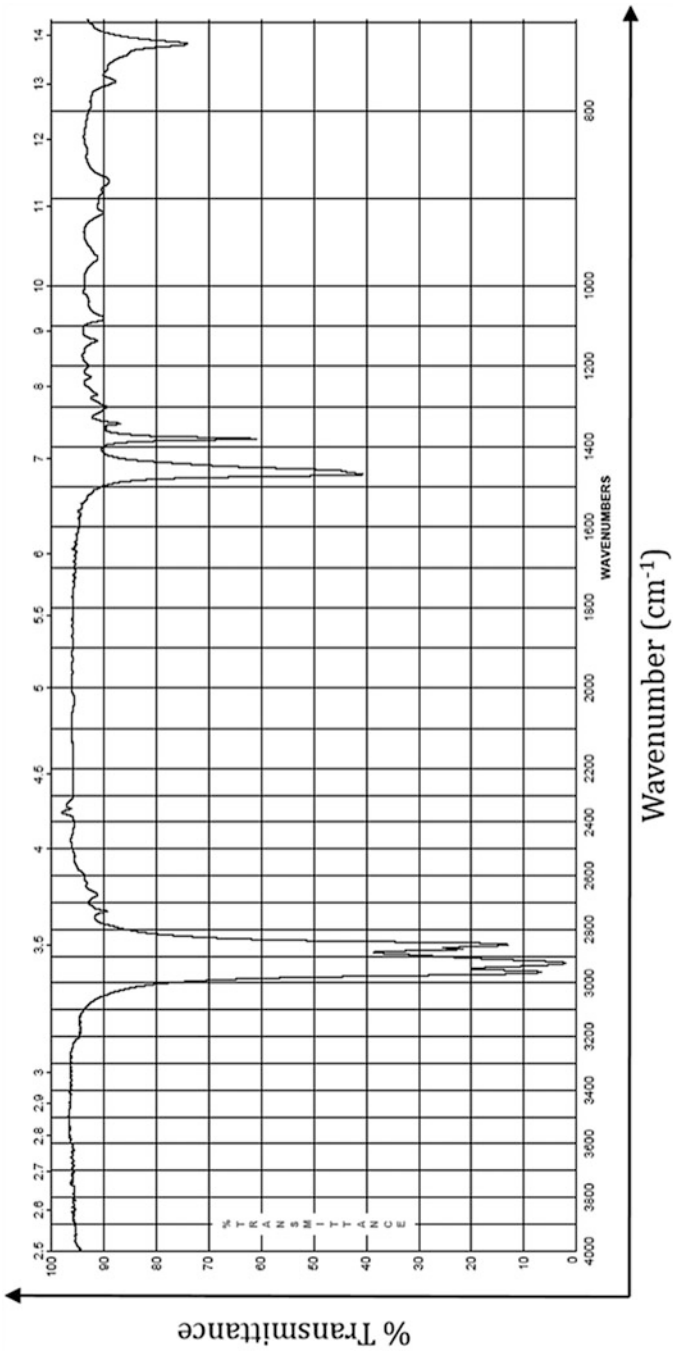


Fig. 4.12 Schematic representation of interpretation of IR spectra

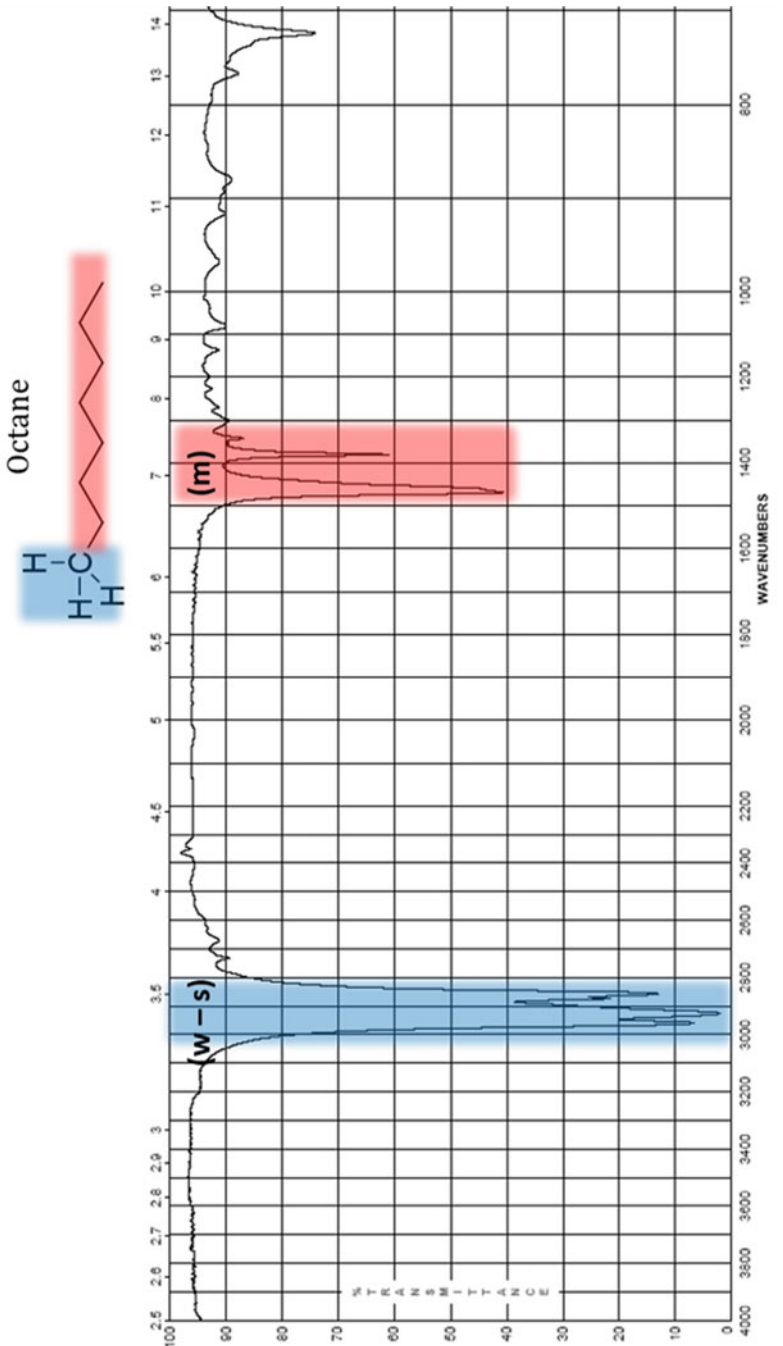


Fig. 4.13 Schematic IR spectra of alkanes

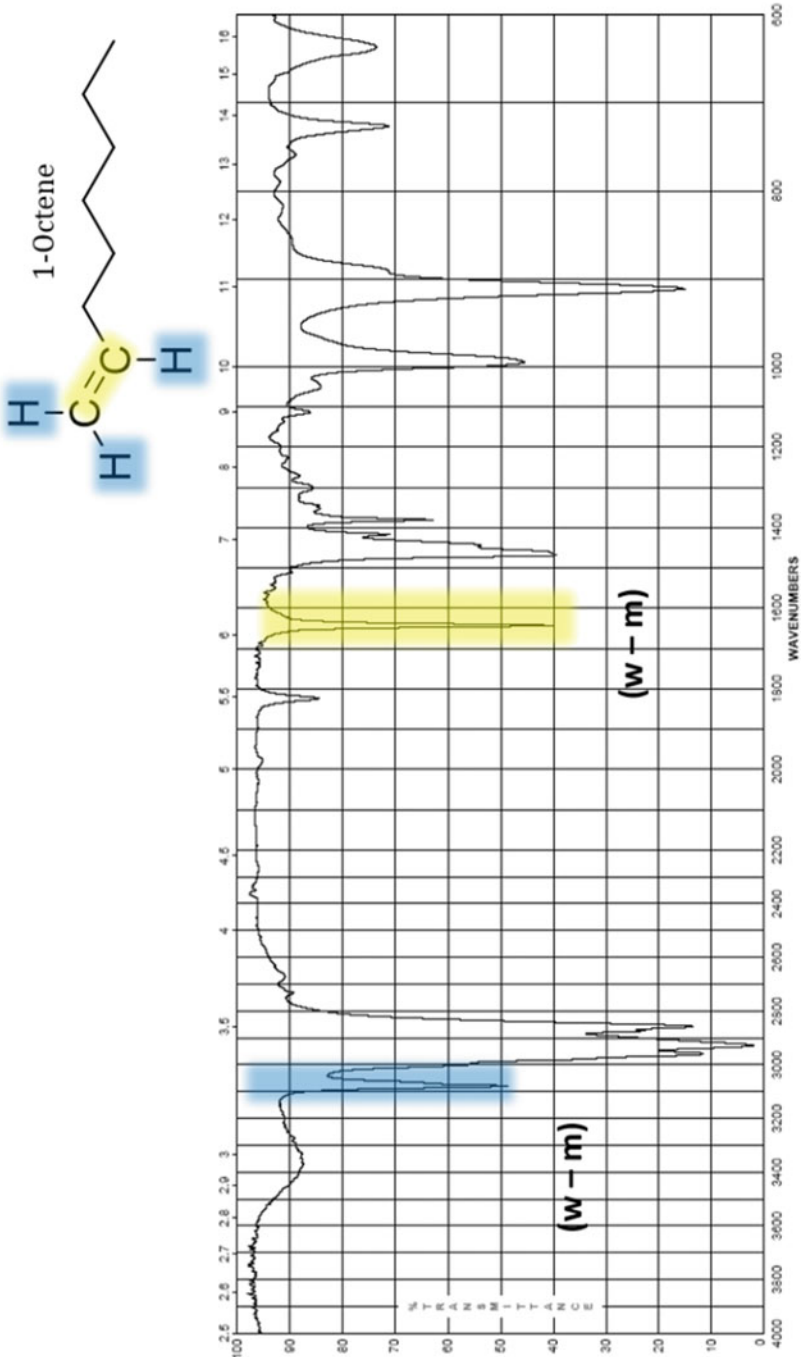


Fig. 4.14 Schematic IR spectra of alkenes

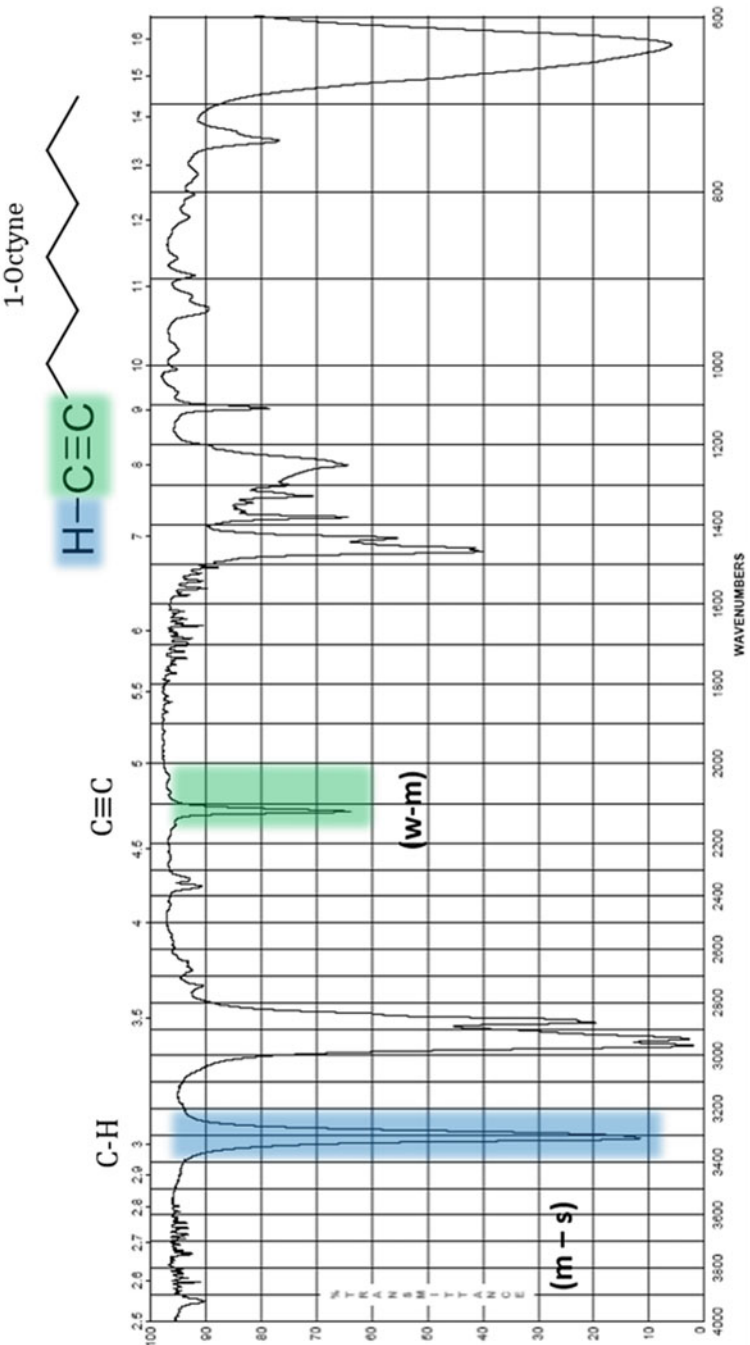


Fig. 4.15 Schematic IR spectra of alkynes

4.9.4 IR Spectra of Aromatic Compounds

Consider the spectra of ethyl benzene (Fig. 4.16). Because of the delocalization of electrons in the ring, the order of C–C bond is 1.5. The energy of stretching frequency for such bonds is relatively low as compared to that of the normal C=C. They are represented as a pair of sharp bands, i.e., 1500 and 1600 cm^{-1} (lower frequency band is stronger). C–H bonds outside the ring appear between 3000 and 3100 cm^{-1} as that of vinyl C–H.

4.9.5 IR Spectra of Ethers

Consider the spectra of di-isopropyl ether (Fig. 4.17). In di-isopropyl ether, addition of C–O–C asymmetric bond and vinyl C–H bonds shows a strong band for antisymmetric C–O–C stretch at 1050–1150 cm^{-1}.

4.10 Applications of IR Spectroscopy

4.10.1 Medical Diagnosis

IR spectroscopy is used for the detection, prevention (early diagnosis), monitoring, and diagnosis (under investigation) of several life-threatening diseases notably cardiovascular diseases, cancers, and diabetes mellitus. As biological samples are of proteins, lipids, carbohydrates, and nucleic acids. All these biochemical substances have unique fingerprints, so any change in them because of the presence disease can be detected using IR spectroscopy.

4.10.2 Identification of Unknown Substance

Small difference in the structure and composition of molecule results in a significant change in the peaks of spectra in specific region. To compare spectrums of unknown substances, computer search systems are required. IR instruments do not offer computer search systems to identify the unknown compounds from the stored IR spectral data. Instead, the position and magnitudes of the peaks in the spectrum of unknown substance are compared with the profiles of pure compounds stored in the library of the computer search system. Computer then matches the profiles similar to that of the analyte and result is displayed (Fig. 4.18). It is very important that sample and the reference should be analyzed in the same conditions such as same physical state, temperature, solvent, etc. One of the main disadvantages is that enantiomers cannot be distinguished because their spectra are identical to each other.

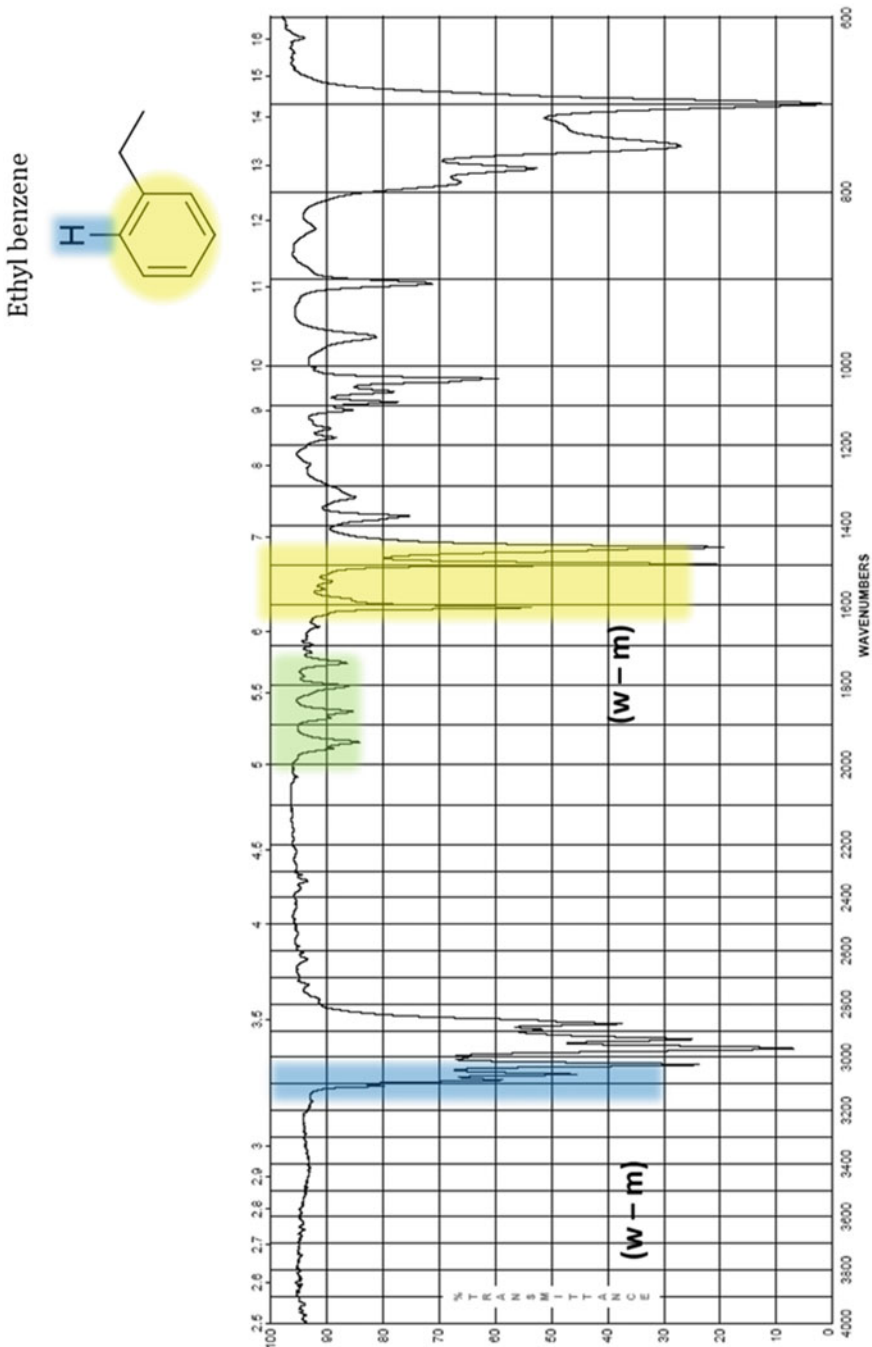


Fig. 4.16 Schematic IR spectra of aromatic compounds

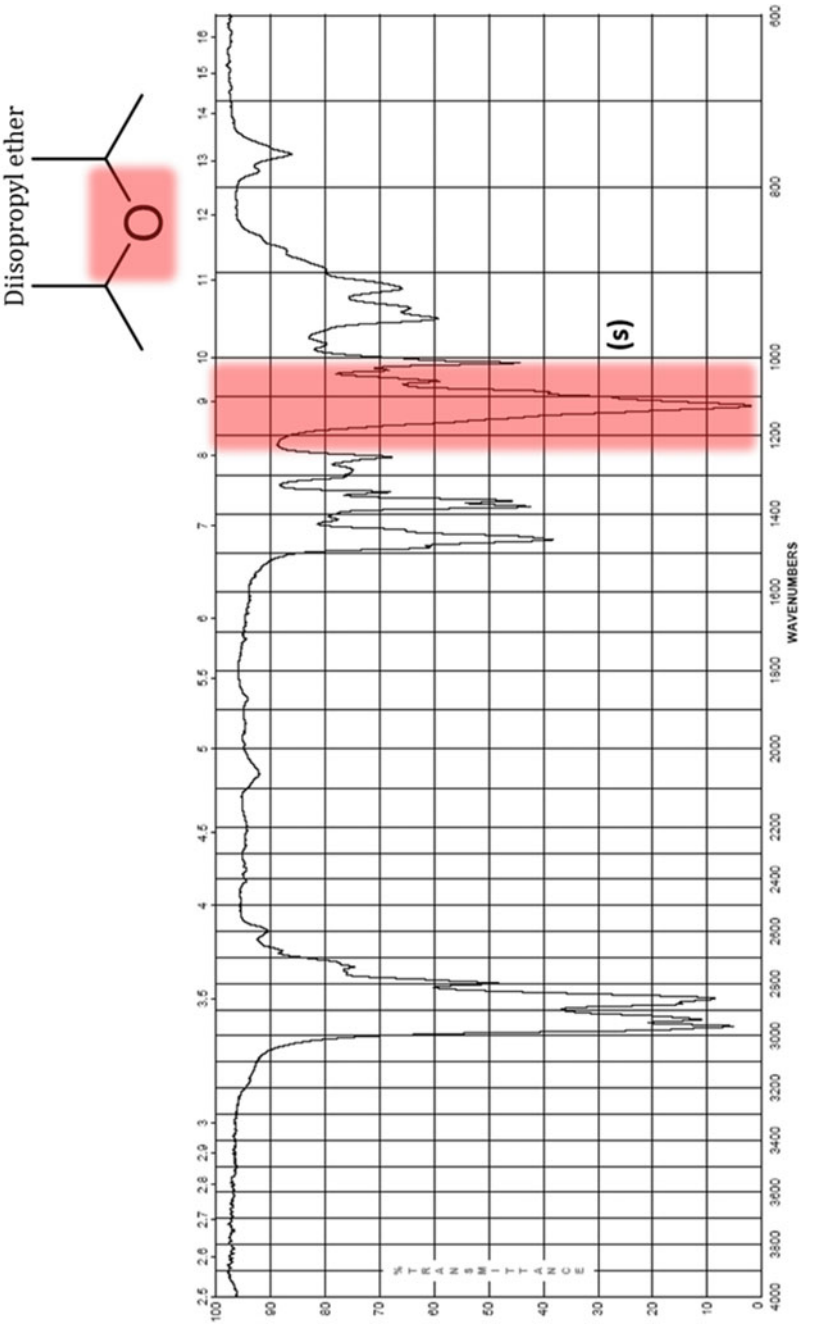


Fig. 4.17 Schematic IR spectra of aromatic ethers

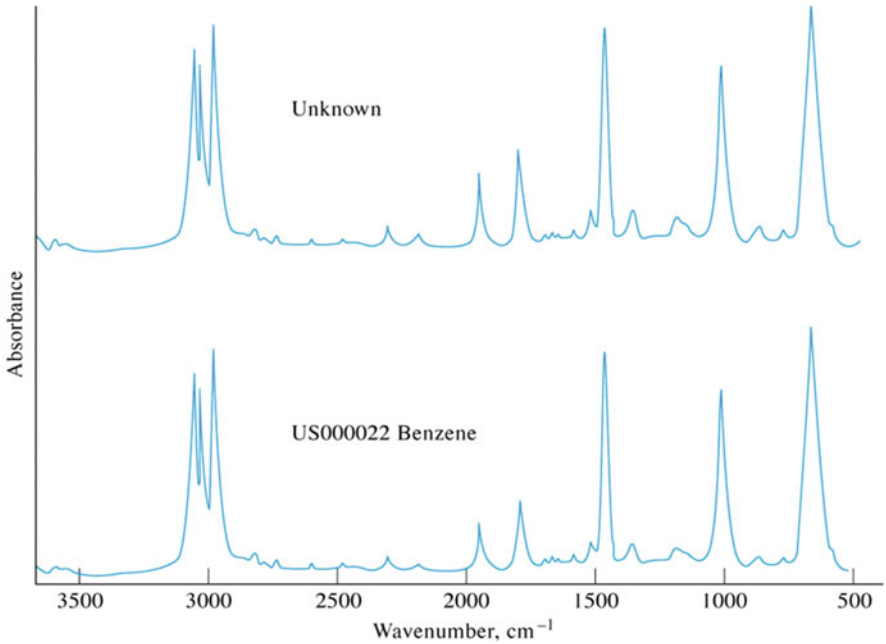


Fig. 4.18 Schematic representation of comparison of IR spectra of unknown compound with that of known one

4.10.3 Determination of Molecular Structure

Identification is carried out on the basis of position of the absorption bands present in the spectrum. For example, C=O at 1717 cm^{-1}. Therefore, absence of the band associated with a particular group points out the absence of that particular group in given compound.

4.10.4 Studying the Progress of Chemical Reaction

IR spectroscopy is used to observe the rate of reaction. It can be done by predicting the disappearance of particular absorption band in the reactants. This shows the utilization of that species and an increase in the rate of the absorption bands in the product shows the formation of particular product. For example, O–H appears at 3600–3650 cm^{-1}, C=O appears at 1680–1760 cm^{-1}.

4.10.5 Detection of Impurities

Presence of impurities in specific compound can be determined by comparing the sample spectrum with the spectrum of pure reference compound. Detection is favored when impurity possess a strong band in IR region where the main substance does not possess a band. For example, the presence of ketone (as impurity) in alcohols can be determined by IR spectroscopy.

4.10.5.1 Detection of API in Final Dosage Form

IR spectroscopy is used to determine the % content of required ingredient and/or API in the final dosage form.

4.10.5.2 Biosimilar and Bioequivalence Studies

Biosimilar and bioequivalence studies of the pharmaceuticals of different competitors can be performed and compared with IR spectroscopy.

4.10.5.3 Determination of Drug–Polymer Interaction

IR spectroscopy notably FT-IR spectroscopy can be used to determine the drug–polymer interaction in the final dosage form.

4.10.6 Analysis of Isomers

IR spectroscopy can be used to find the following types of isomers of the compounds.

4.10.6.1 Geometrical Isomerism

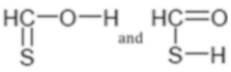
Trans-isomer gives a simpler spectrum than the cis-isomer due to the presence of symmetrical carbon in structure.

4.10.6.2 Conformers (Rotational Isomers)

They can be identified with the help of high-resolution IR spectrometers.

4.10.6.3 Tautomerism

Presence of two or more chemical compounds capable of interconverting, usually by exchanging a hydrogen atom between the two atoms, can be determined with the help of IR spectroscopy. For example, thiocarboxylic acid.



4.10.6.4 Functional Group Isomerism

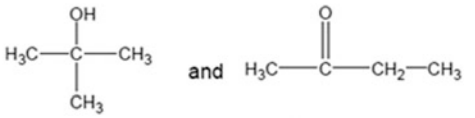
Isomerism shown by the compounds having same molecular formula but different functional groups can also be determined using IR spectroscopy. For example, dimethyl ether (CH_3–O–CH_3) and ethanol (CH_3–CH_2–OH) have same molecular formula, i.e., C_2H_6O but have different functional groups. In dimethyl ether, the functional group is ether (–O–), whereas in ethanol, the functional group is alcohol (–OH). The characteristic peak of –OH group appears at 3500–3100 cm^{-1} which differentiate the ethanol from dimethyl ether.

4.10.6.5 Determination of Symmetrical Shapes of Molecules

With the help of IR spectroscopy, symmetry of the molecules may be determined. For example, nitrogen dioxide (NO_2) if linear in shape then only two bands appear, but if it is bent, then three bands appear. The actual IR spectrum of NO_2 shows three peaks at 750, 1323, and 1616 cm^{-1}. Likewise, IR spectrum is also used to determine the structures of XeF2 (linear in shape), XeF_4 (square planar), and XeF_6 (octahedral).

4.10.6.6 Identification of Functional Groups

IR region can be divided into two regions, group frequency region (4000–1500 cm^{-1}) and fingerprint region (1500–400 cm^{-1}). Group frequency region gives the determination of functional group, whereas fingerprint region can be used to identify the atom in molecule.



2-methyl-2-propanol (3500-3100 cm^{-1})

2-butanone (1700 cm^{-1})

4.10.6.7 Study of Inorganic Complexes

During the complex formation, large number of new bonds formation occurs which can be easily detected by IR spectroscopy.

4.10.6.8 Detection of Moisture in Samples

In the presence of lattice water, spectra will comprise of three characteristic bands at 3600–3200 cm^{-1}, 1650 cm^{-1}, and 600–300 cm^{-1}.

4.10.6.9 Characterization of Heterogeneous Catalysts

It is useful to determine the nature of molecules attached with catalyst surfaces. For example, characterization of olefin polymerization catalysis with silica gel.

4.10.6.10 Forensic Analysis

Polymer degradation can be analyzed in both criminal and civil cases. For example, it is used to determine the blood alcohol content in suspected drunk driver.

4.10.6.11 Analysis of Multilayered Polymeric Film

Identities of polymer materials in the multilayered film can be determined with the help of IR spectroscopy.

4.11 Advantages

1. Easy to use.
2. Inexpensive.
3. Analysis takes <10 min.
4. IR spectrophotometry can eliminate the common possible errors that appear in other spectrophotometric techniques.
5. Same sample cell is utilized for all kind of determinations.
6. All measurements are done based on the defined spectrum without dependence on λ intensity.

4.12 Disadvantages

1. Sample cell is made up of alkali halides; therefore, it is very sensitive to the absorption of water.
2. In case of excessive amount of moisture, penetration distance of absorbing light decreases.

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Atomic Spectroscopy

5

Abstract

Atomic spectroscopy is a type of spectroscopic technique which is used for both quantitative and qualitative analysis of an element present in sample through mass spectrum. Atomic spectroscopic technique has two techniques; atomic absorption spectroscopy (AAS) and atomic emission spectroscopy (AES) which involve the energy absorption in the process of excitation or energy emission in the process of decay, respectively. AAS and AES have been briefly described in Chaps. 6 and 7, respectively. In this chapter, we have focused on the basic mechanism of atomic spectroscopy along with its advantages, disadvantages, and applications.

Keywords

Types of atomic spectroscopy · Atomic absorption spectroscopy · Atomic emission spectroscopy

5.1 Introduction

In an atom, electrons are arranged according to their energy levels. They are arranged in sub-shells; the sub-shells are arranged in shells and shells are arranged around the nucleus. Electrons near the nucleus have lower energy level (also known as ground state) than the electrons that are much far away that have high energy level (also known as excited state). However, they experience stronger attraction in the nucleus than those ones that are further away from the nucleus. Ground state is a status where the atom's electrons are in their lowest possible energy level (stable). Excited state is another status where the atom's electrons absorb enough energy to be promoted to a higher level (Fig. 5.1). Therefore, they are not in their lowest energy level (unstable). Generally, atoms are in their "ground state" but when an atom receives enough input of energy that their electrons require to be promoted to a higher energy level, they will then turn to their "excited state." Since, an atom's

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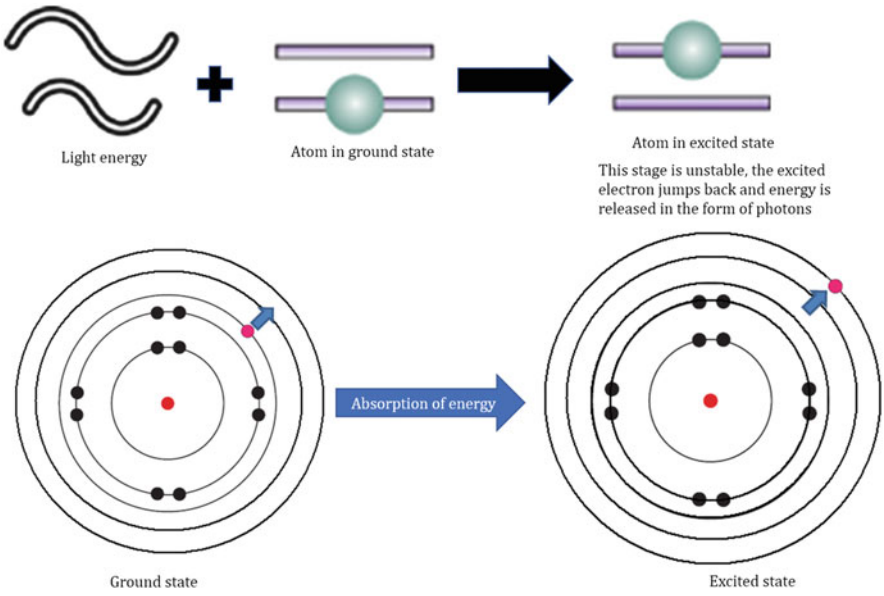

Fig. 5.1 Schematic representation of absorption and excitation of energy by the electron in atomic spectroscopy

excited state is very unstable, it rapidly "jumps" back down to its ground state. This "jump" then causes the atom to release the energy it absorbed in the form of photons of light.

5.2 Principle

Samples containing metals of unknown concentration can be analyzed using both types of spectroscopic techniques, i.e., AAS and AES. Both techniques possess the same instrumentation. The only difference is that in AAS, light absorbed by the excited atoms or ions of the metal whose concentration is going to be measured is measured, whereas in AES, the light emitted by the excited atoms or ions of the metal whose concentration is going to be measured is measured. In AAS, when a light of certain wavelength passes through an atom or ion, it excites that atom or ion from ground state (having low energy level) to an excited state (having high energy level). As the number of atoms or ions of the metal of interest present in sample is increased in the path of light, the amount of light absorbed is also increased and this wavelength is directly proportional to the concentration of absorbing atoms or ions of metal of interest. As the wavelength of the light absorbed is specific for each element, the measurement of this absorbed light gives a quantitative measurement of the amount of analyte present in the sample. In AES, the sample is subjected to high energy thermal environment to produce the excited state which can emit the light. As

the wavelength of light emitted by atoms or ions of metal of interest is specific for each element present in the sample, so, the measurement of this emission spectrum which is the collection of emission lines from the excited atoms gives a qualitative measurement of the analyte present in the sample. As the number of atoms or ions is increased, the amount of the emission intensity will also be increased.

5.3 Types of Atomic Spectroscopy

Following are the main types of atomic spectroscopy:

1. Atomic absorption spectroscopy: This technique has been briefly described in Chap. 6.
2. Atomic emission spectroscopy: This technique has been briefly described in Chap. 7.

5.4 Advantages

1. High accuracy and sensitivity.
2. Accurate precision.
3. Easy to use.
4. Helpful in detecting the trace elements in the presence of higher concentration of other elements.
5. Can reach previously inaccessible places.

5.5 Disadvantages

1. Used to determine ppm levels of metals, so could not be used for the analysis of light elements, e.g., carbon, hydrogen, oxygen, nitrogen, phosphorus, sulfur, halogens, and noble gases.
2. Only liquid samples can be detected, hence solid samples need vaporization prior to detection.
3. Samples need dilution prior to their determination.
4. Another drawback is that flame atomization produces both atoms and ions but only atoms are detected through this technique because atoms which undergo ionization do not undergo atomic absorption.
5. Atomic spectrophotometer is costly.

5.6 Applications

1. More or less than 68 elements can be determined using this technique with good precision. Therefore, in pharmaceutical industry, this technique is helpful for the analysis of active pharmaceutical ingredient (API), raw material, or intermediates used for drug development.
2. In pharmaceutical industry or drug testing labs, these techniques are used to determine a metal catalyst that can be present in a drug after purification, e.g., detection of trace elements in multivitamin formulations, zinc in insulin, cobalt in vitamin B_{12}, lithium in anti-depressants, etc.
3. In medical laboratory, both AAS and AES can be used to analyze the tissue samples for the detection of type and amount of toxic metals. Tissue samples may be blood, urine, hair, bone marrow, or nails. A typical example is the measurement of electrolyte sodium or potassium in the plasma.
4. These techniques have also found their importance in mining industry as they can be used for quantitative and qualitative determination of precious metals like silver, gold, etc.
5. Water analysis can also be done using atomic spectroscopy which may include drinking water, sewerage water, or marine water, e.g., analysis of leaching of zinc and lead from tin-lead solder in water.
6. An oldest application of atomic spectroscopy is the analysis of animal products, animal feeds, and vegetables in food industry. Food samples are analyzed to detect the mineral and trace elements.
7. As this process relies on atomic absorption or emission, it can reach previously inaccessible places. For example, miners use this technique to determine whether the rock contains enough metals or not.

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Atomic Absorption Spectroscopy

6

Abstract

In this chapter, we have discussed the components of AAS. The two types of AAS and their comparison have been discussed in detail. The comprehensive method for working of AAS has also been elaborated here. Moreover, we have also discussed the various types of interferences that may influence the results of AAS. Applications of AAS along with precautionary measures, advantages, and disadvantages have also been discussed here.

Keywords

Components of AAS · Hollow cathode lamp · Electrodeless discharge lamp · Total consumption burner

6.1 Introduction

Atomic absorption spectroscopy (AAS) is a spectroscopic technique which is widely used for elemental analysis. The basic principle involved in AAS is the absorption of energy by the atoms at ground state in the gaseous state and the amount of light absorbed by the atom at ground state is determined. Once the amount of light absorbed by the atom at ground state is determined, we can estimate the unknown concentration of metal accordingly.

In 1952, Australian scientist Alan Walsh was working on the measurement of small concentrations of metals using atomic emission spectroscopy (this technique has been discussed in detail in Chap. 7). The idea of AAS came into his mind as he was gardening at his Melbourne home. On the normal Sunday morning, he had the idea about looking at the light absorbed by the atoms except than looking at the light they emit. Alan Walsh did not just discover a process that has the ability to save lives, but also proven that atoms will only absorb light that has the exact value requires to promote their electron to a higher level. AAS is a technique in which radiations are absorbed by non-exited atoms in vaporized form. This technique is

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used to determine the amount of several metals (e.g., Cu, Fe, Zn, Mg) in soil, blood, urine, air, water, food, etc. In electromagnetic spectrum, AAS uses the visible part of electromagnetic radiation to detect the presence of metals present in sample. The main energy source used in this technique is hollow cathode lamp and electrodeless discharge lamp. AAS is especially used for the estimation of the trace metals in samples independent from the molecular form of the interested metal which is present in the sample. For example, we can also estimate the total content of metal present in the water sample either that metal exists as salt form such as chloride and sulfate.

6.2 Principle

When a sample having metallic species is placed into a flame, the vapor of metallic species is formed. The phenomenon behind is that the atoms of a specific element in ground state absorb the radiation of light of their own specific wavelength and jumped up from low energy state (ground state) to the higher energy state (excited state). The absorption of light energy that has the right wavelength to be absorbed by the metal, promotes the electrons of metal from the sample to jump up from a lower energy level (ground state) to a higher energy level (excited state) as clearly shown in Fig. 6.1.

In AAS, two processes and/or phenomenon are involved that are as follows:

1. When light energy is absorbed by the metal sample, free atoms are produced from the sample metal but are not ionized.
2. Once the free atoms are produced, they further absorb radiation from an external source.

These free atoms once produced in the flame after absorption of radiation cause a transition of these atoms from the ground state (having low energy level) to an excited state (having high energy level after absorption of light). The amount of light absorbed by specific element present in the sample is directly proportional to the density of atoms present in the flame and here, AAS determines how much light

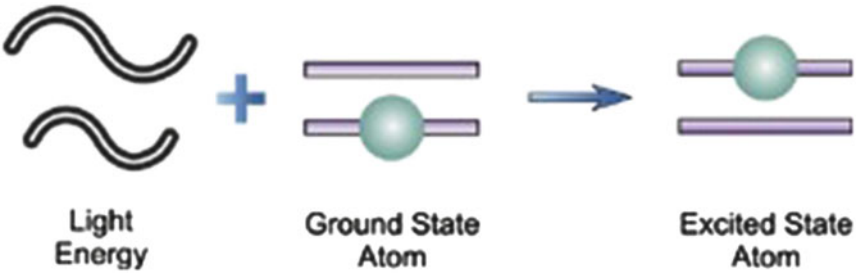

Fig. 6.1 Schematic representation of excitation of atom after absorption of light

absorbed by the sample. Once it is determined that how much light energy was absorbed by the atom of analyte present in sample, the concentration of metal in the sample can be estimated. The total amount of the light of specific wavelength absorbed by the sample solution is calculated using the following equation:

$$\text{Total amount of light absorbed by the metal present in sample} = \left(\pi e^2/mc\right)Nf$$

where e = charge on the electron, C = speed of light, N = the total number of atoms, f = strength of oscillator.

6.3 Components of AAS

A schematic diagram of AAS has been shown in Fig. 6.2 which contains the following components:

6.3.1 Radiation Source

The radiation source is one of the main components of AAS. It is of two types. The detail description of these radiation sources is as follows:

6.3.1.1 Hollow Cathode Lamp (HCL)

In HCL, the cathode has a hollow cup. The sample which contains metal whose concentration is to be determined is placed in the cup of HCL. In HCL, anode is made up of tungsten wire. In a tube, an inert gas is placed between the two electrodes. The lamp window is made up of quartz, silica, or glass (Fig. 6.3). When a voltage is applied between the two electrodes, the inert gas becomes partially ionized at the anode and moves towards cathode. As a result, cathode vaporizes metal atoms present in it and gives rise to spectrum of that metal. In the overall phenomenon, the pressure of lamp is important which should be kept from 1 to 5 Torr, because ionization at the low filling pressure remains largely confined to the interior of the cathode. The noble gas ions, traveling toward the cathode, start to

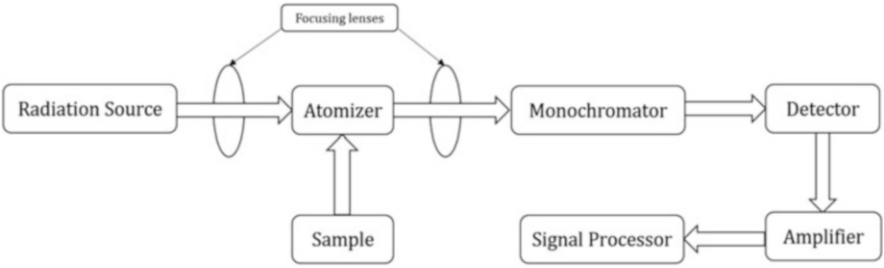

Fig. 6.2 Schematic representation of components of atomic absorption spectroscopy

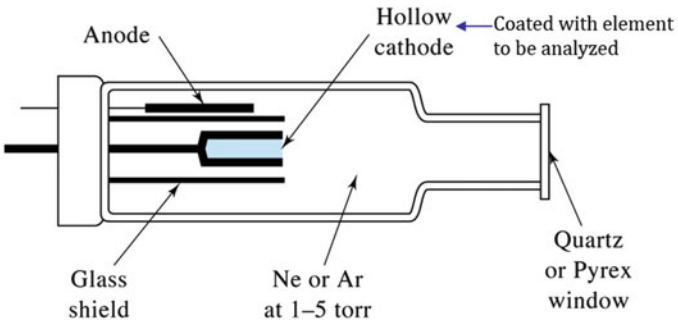

Fig. 6.3 Schematic representation of hollow cathode lamp

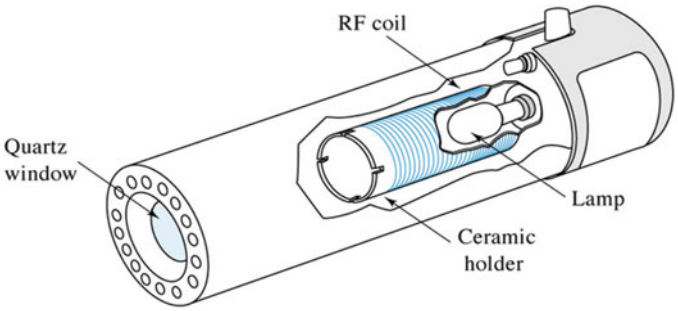

Fig. 6.4 Schematic representation of electrodeless discharge lamp

strike the interior of the cathode thereby sputtering off atoms of the metal composing it, in sufficient numbers to give rise to a cloud of metallic atoms. The spectral lines produced in HCL after the absorption of light by the metal. The spectrum lines emitted by HCL of the metal represent the type of metal that is present in the cathode. For example, the spectrum of copper is obtained from the emission of copper cathode lamp which is then absorbed by the copper atoms present in lamp.

6.3.1.2 Electrodeless Discharge Lamp

One of the main drawbacks of using HCL is that it is very difficult to construct it from those elements that are volatile in nature, for example, germanium and arsenic. To overcome this drawback, an alternative source of light has been introduced that is known as electrode discharge lamp (EDL). EDL is an evacuated tube (Fig. 6.4) and the metal whose concentration is to be determined from the sample is placed in an evacuated tube. The tube contains argon whose pressure is kept low and sealed off. When the sealed tube is kept in a microwave discharge cavity, the argon is shifted towards a plasma state which is responsible for the excitation of metal that is sealed inside the tube. The emission of radiation from the metal is a spectrum of the metal present in it.

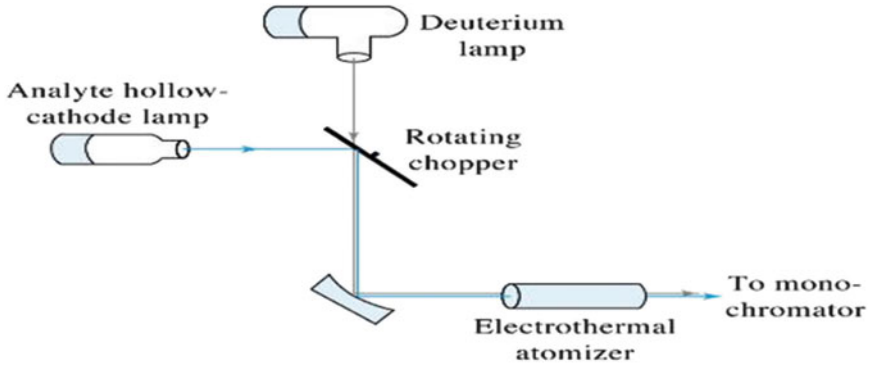

Fig. 6.5 Schematic representation of working of chopper

6.3.2 Chopper

Chopper is just like a rotating wheel which is placed between the radiation source and the flame. The reason to use chopper is that when the steady light comes from the lamp, chopper converts it in to a pulsating light (Fig. 6.5). As a result, the pulsating current is produced in photocell. The emitted light from the flame produces a steady current. The steady current and pulsating current both are present but only pulsating current is amplified and recorded by read-out device.

6.3.3 Atomizers

Atomizers are the devices that are responsible to carry out the phenomenon of atomization. Atomization is actually the phenomenon of separation of atoms and/or molecules present in sample into individual molecules or atom and breaking molecules into the atoms of metal whose concentration is going to be determined. Atomization is a process in which the free atoms of the metal of interest whose concentration is going to be determined are produced by heat. This can be done by exposing the analyte present in sample, in flame at high temperature. There are two types of atomizers used in AAS.

6.3.3.1 Flame Atomizers

This is common method in which flame is used. The liquid sample which contains the metal of unknown concentration or whose concentration is going to be determined is converted into the gaseous state by using the flame. The flame is also responsible for the conversion of molecular form of the metal into its atomic form which can be achieved at vapor state. In flame atomizers, two types of burners are usually used and their details have been described in the following sub-sections:

Total Consumption Burner

In this type of flame atomizer, the sample solution which contains metal of unknown concentration, oxidizing gases, and the fuel is allowed to pass through separate passages to meet at the opening of the base of flame as shown in Fig. 6.6. The mixture of hydrogen or acetylene with oxygen is used in this type of burner and it gives rise to intense hot flames. The sample is present in the liquid form which is broken up by the flame and is converted into the droplets which are then evaporated and ultimately burns by leaving behind the residues which are reduced into atoms of the metal of unknown concentration present in sample solution.

Regions of the Flame in Burner

Following are the three main regions of the flame in total combustion burner (Fig. 6.7).

Primary combustion zone: Thermal equilibrium cannot be obtained in this region. Therefore, it is seldom used for spectroscopic analysis. This region exists at the tip and/or opening of the flame.

Interzonal combustion region: This region is situated between the primary and secondary combustion zone. This region is relatively narrow region in the flames of

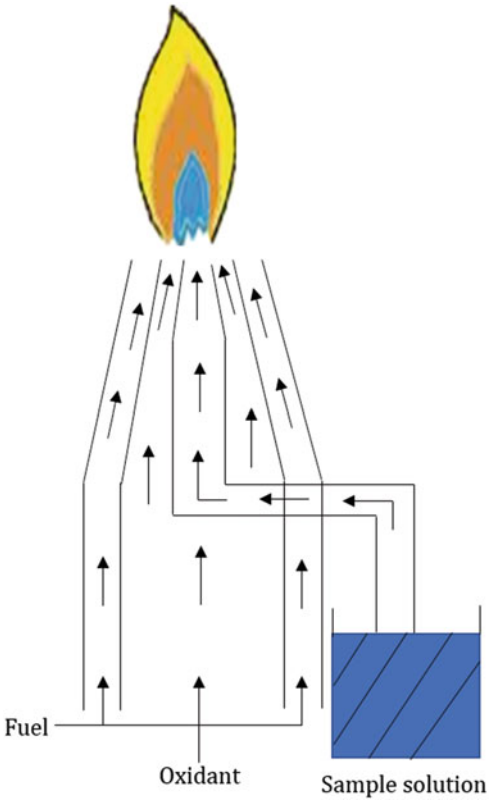

Fig. 6.6 Schematic representation of total combustion burner

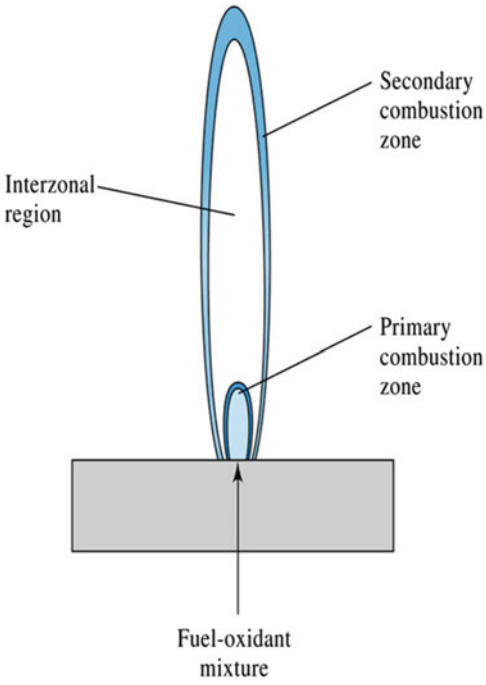

Fig. 6.7 Schematic representation of regions of the burner

burner and often rich in free atoms of the metal of unknown concentration. This region is the hottest part of the flame and is widely used in spectroscopic analysis of the metals of unknown concentration.

Secondary combustion zone: In this region of flame, the species of metal of unknown concentration are converted into stable molecular oxides and then dispersed into the surroundings of the flame. This part of flame is also seldom used in spectroscopic analysis of the metals.

Premixed Burner

In this kind of burner, the fuel gas and oxidants cause the aspiration into a large chamber. This is carried out under the pressure. The fine droplets of the premixed fuel and oxidants along with larger drops of the sample that contain the metal of unknown concentration are collected at the outlet of the chamber and then introduced into the flame. The excess amount is then drained out.

6.3.4 Nebulization

Nebulization is the phenomenon in which the liquid sample is converted into the fine droplets before entering of the liquid sample into the burner. This process is used for the formation of small and fine droplets. The conversion of liquid sample into fine droplets is known as nebulization. This is the common method of nebulization in

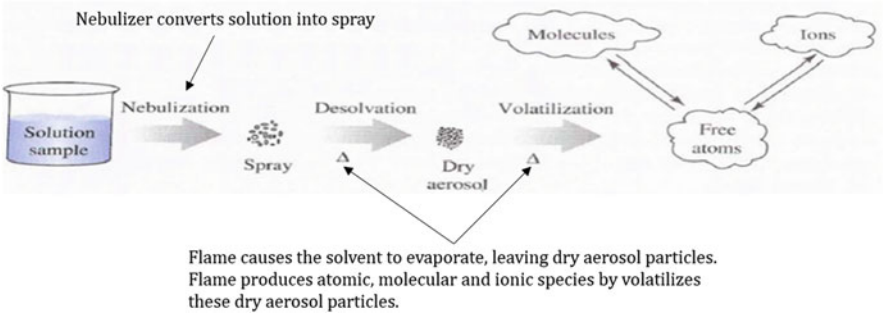

Fig. 6.8 Schematic representation of nebulization phenomenon in atomic absorption spectroscopy

which a gas is used having a high velocity, called pneumatic nebulization. A schematic representation of nebulization phenomenon has been described in Fig. 6.8.

6.3.5 Monochromators

Monochromator is an essential part of AAS. It is used for the separation of all the thousands of spectral lines into individual ones. If good monochromator is not used, the efficiency of AAS is reduced because of the limit of detection of individual spectral lines of metallic species is severely compromised. Monochromator uses the light of specific wavelength with respect to the metal of unknown concentration. The sample which contains the metal of unknown concentration absorbs the light of specific wavelength and excludes all the other wavelengths. That specific light coming from the monochromator permits the detection of the interested element and/or metal whose concentration is going to be determined even in the presence of others elements.

6.3.6 Detectors

These are the devices that are used to detect which wavelength is being absorbed by the sample having the metal whose concentration is going to be determined.

6.3.7 Amplifier

It amplifies the signals received from the detector and then transfers to the read-out device.

6.3.8 Read-Out Device

The most widely used read-out devices in AAS are as follows:

1. Digital voltmeter
2. Simple galvanometer
3. Potentiometer
4. Computer

6.4 Working of AAS

Before the use of spectrometer, it should be calibrated by standard solution of known concentration of solute which has to be determined in the test solution. Cuvettes are filled with standard solution or reference solution and placed in sample holder in spectrometer. The light of specific wavelength is directed towards the sample and/or reference solution. Before reaching of light ray towards the sample or blank, the light will pass through a series of prism, diffraction grating, and mirrors. The prism splits light into different wavelengths, the mirror is used for navigation of light and the diffraction grating allows the light of the required wavelength to pass through it and reaches towards the cuvette containing reference or standard solution. It analyzes the reflected light and compare with predetermined standard solutions. Some part of light is absorbed by the solution and remaining part is transmitted towards the detector. The detector measures the intensity of transmitted light and converts it into electrical signals. These electrical signals send to the read-out device (galvanometer). The galvanometer measures electrical signals and displays in digital form. This digital representation is actually the absorbance and/or optical density of the analyte present in sample solution. A schematic representation of working of AAS has been described in Fig. 6.9.

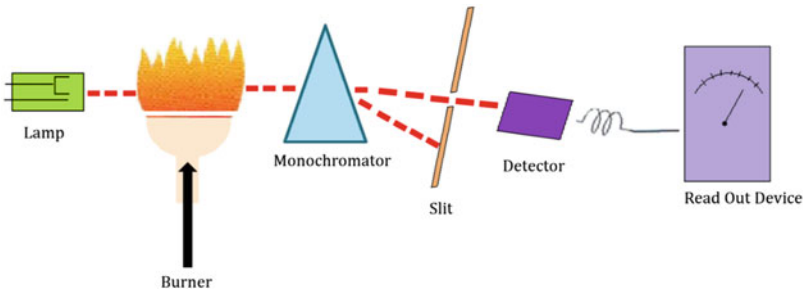

Fig. 6.9 Schematic representation of working of atomic absorption spectroscopy

6.5 Types of AAS

Following are the two main types of AAS:

6.5.1 Single-Beam AAS

The light of wavelength between 325 and 1000 nm is used in single-beam AAS. The light travels from the sample solution and/or reference solution separately (Fig. 6.10). The light source, e.g., HCL, emits the sharp atomic line of the element whose determination is going to be determined. The chopper is interposed between the light source and flame. The light is modulated easily by rotating the chopper located between the light source and the flame and can also be achieved by pulsing the power. Modulation helps to differentiate the light coming from the radiation source lamp for the emission. The modulated light is then led to the flame where the atoms of the element present in sample are in ground state (low energy level) and after absorption of light radiation, these atoms are jumped to the excited state (high energy level). The monochromator, which separates the wavelength of interest, is then led to the detector followed by read-out device.

6.5.2 Double-Beam AAS

The light of the wavelength between the range of 185 and 1000 nm is used in double-beam AAS. It has two cells which can be used separately for the sample solution and the reference one. The light coming from the monochromator is split by an instrument which is known as splitter into two beams. One of the two beams is used for the reference and other one is used for the sample solution (Fig. 6.11). The chances of error are eliminated in double-beam AAS which may occur due to fluctuations in light output and sensitivity of detector.

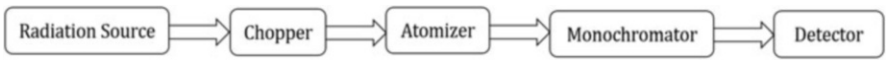

Fig. 6.10 Schematic representation of single-beam atomic absorption spectrophotometer

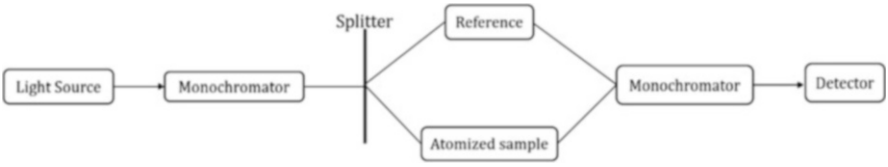

Fig. 6.11 Schematic representation of double-beam atomic absorption spectrophotometer

Table 6.1 Comparison between single and double beam atomic absorption spectrophotometer

Single-beam AAS	Double-beam AAS
It is simple in working	Its working is complicated
It is less expensive	This equipment is costly
In single-beam AAS, the automation is low	While in double-beam AAS, the speed of automation is high
The efficiency of light is more in single-beam in AAS	The efficiency of light is less in double-beam AAS
Due to single beam in AAS, reference and sample are rung separately and more time is consumed to analyze the sample	Due to the presence of double beam, reference and sample are rung simultaneously and less time is required for analysis
Single-beam AAS has low stability because it depends upon the intensity of single beam	Double-beam AAS has maximum stability because it depends on the ratio of intensity of light between the two beams
Due to the presence of single beam, the chances of fluctuation in the intensity of light and reading of data are more	There are lesser chances of fluctuation in the intensity of light and readings of data as separate light beam is used for the reference and the sample solution
Due to single beam, the reference and the sample are placed separately	As double beam is present, the reference and the sample are kept at the same time
In single-beam AAS, the light throughput is high and more resolution is attained	In double-beam AAS, the light throughput less and decreased resolution is attained
Single-beam AAS takes time to warm-up and takes time to start analysis	Double-beam AAS does not take much time to warm-up and ready for analysis

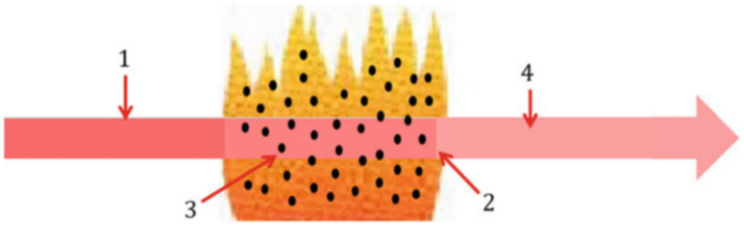

Fig. 6.12 Schematic representation of collection of data from atomic absorption spectrophotometer

6.5.2.1 Difference Between Single- and Double-Beam AAS

The comparison between single-beam and double-beam AAS has been described in Table 6.1.

6.6 How Can We Obtain the Data of AAS?

Consider the following Fig. 6.12, we can obtain the data by the following way:

Number 1 mentioned in Fig. 6.12 reflects the intensity of light coming through the radiation source is measured. Number 2 mentioned in Fig. 6.12 indicates that the

light can then be absorbed by the atoms of the metal whose concentration is going to be determined that has been vaporized in the flame. This wavelength can cause the transition of the electrons that are present in ground state (low energy level) into a higher energy level (excited state). Number 3 mentioned in Fig. 6.12 indicates that the higher the concentration of the metal present in the sample, greater will be the absorbance of light. Number 4 mentioned in Fig. 6.12 indicates that the intensity of light after passing through the sample which contains the metal of unknown concentration is measured again and compared with the first result that was obtained with blank and/or reference.

6.7 How Do We Analyze the Data of AAS?

The data can be analyzed by comparing the light intensity absorbed by the sample which contained the metal of unknown concentration with blank and/or the reference at the same time. For the construction of calibration curve, it is important to measure the absorbance of different known concentrations of the metal whose unknown concentration present in samples is going to be determined. The instrument is calibrated using several solutions of known concentrations of the metal. The absorbance of each known solution is measured. In calibration curve, the absorbance obtained by the spectrophotometer is plotted against its known concentration (Fig. 6.13a). Through the calibration curve, we can then determine the unknown concentration of metal present in the sample. Once the calibration curve is constructed, it can be used to determine the unknown concentration of an element in a solution. We can determine the unknown concentration of analyte with the help of this calibration curve. The sample solution that contains metal of unknown concentration is introduced into the flame of AAS. The metal present in sample absorbs the radiation of light having specific wavelength that is measured with the help of detector and interpreted by read-out device. The unknown concentration of the element is then estimated from the calibration curve which is obtained by comparing the absorbance of unknown concentration with that of absorbance of the known concentration as represented in Fig. 6.13b.

6.8 Interferences of AAS

Interferences can be defined as increasing or reducing the extent of absorption of light due to any physical or chemical interference. It is achieved with the test element in aqueous solution. In the sample, the element other than the one of interested element may absorb the radiation of specific wavelength which is being used for specific element. Following are the most commonly observed interferences when AAS is used:

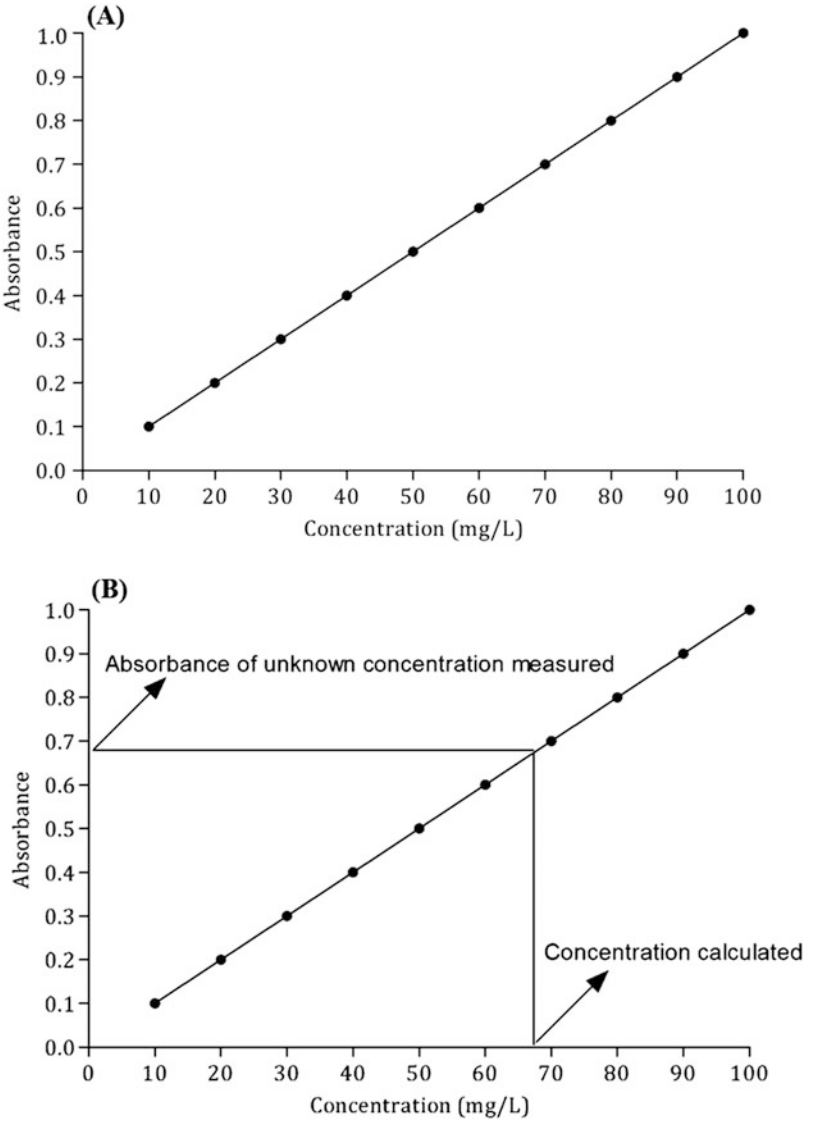

Fig. 6.13 Schematic representation of calibration curve for known concentrations of standard solution (**a**) and calculation of concentration of unknown analyte (**b**) with the help of standard curve

6.8.1 Ionization Interference

The absorption of radiation is low due to the formation of ions rather than atoms. To overcome this problem, ionization suppressors are usually added to prevent the formation of ions during the combustion of sample solution in flame.

6.8.2 Back Ground Absorption of Source Radiation Interference

Due to incomplete atomization, presence of particles causes the absorption of background source radiation. This problem can be overcome by increasing the temperature of flame.

6.8.3 Transport Interference

Rate of nebulization, viscosity, density, vapor pressure, surface tension and rate of aspiration of the sample are the factors that may also affect the results of AAS.

6.8.4 Cation–Cation Interference

Sometimes, the signal intensity of the interested element present in the sample is irregularly decreased. These are neither the ionic nor the spectral in nature and their interactions mechanism is unknown.

6.8.5 Anion–Cation Interference

The intensity of radiation that is emitted by an element is affected due to the presence of certain anions in the sample solution and thus causes a serious analytical error.

6.8.6 Oxide Formation Interference

If oxygen is present in the flame, then the stable oxides with free metals are produced that are responsible for this kind of interference. The intensity of emitted radiation is lowered due to the removal of a large percentage of free metal atoms from the flame. The oxides of alkaline earth metals are subjected to this kind of interference.

6.8.7 Spectral Interferences

It includes spectral overlap, molecular absorption, and light scattering. Few examples of the spectral interferences of some metals are presented in Table 6.2.

6.8.8 Chemical Interferences

It may include thermal stability and ionization ability of the molecules present in the sample.

Table 6.2 Examples of spectral interferences of interfering elements with that of target elements

Target element	Spectral line (nm)	Interfering element	Spectral line (nm)
Aluminum (Al)	308.215	Vanadium (V)	308.211
Calcium (Ca)	422.673	Ge	422.657
Cadmium (Cd)	228.802	Arsenic (As)	228.812
Cobalt (Co)	252.136	Indium (In)	252.137
Copper (Cu)	324.754	Eu	324.753
Iron (Fe)	271.903	Platinum (Pt)	271.904
Ga	403.298	Mn	403.307
Mercury (Hg)	253.652	Cobalt (Co)	253.649
Mn	403.307	Ga	403.298
Sb	217.023	Lead (Pb)	216.999
Si	250.690	Vanadium (V)	250.690
Zn	213.856	Iron (Fe)	213.859

6.8.9 Physical Interferences

It includes viscosity, surface tension, and density of the sample solution that contains metal of unknown concentration.

6.8.10 Vaporization Interferences

The rate of vaporization of the salt particle present in the sample solution of metal whose concentration is going to be determined may alter due to the presence of some component in the sample solution.

6.9 Applications

1. It is one of the most widely used techniques for qualitative analysis of the metals.
2. It is also used for quantitative analysis of the metals even they are present in the trace amounts.
3. It can also be used for simultaneous multicomponent analysis but this can be done by using multicomponent hallow cathode lamp.
4. *Detection of metals in biological fluids*: It is used for elemental profiling in biological fluids such as in urine and blood samples.
5. *Determination of metallic elements in food products:* Metallic element such as copper, nickel, and zinc are the most common toxic elements that may be present. AAS can be used to detect any of these metals from the raw material that is used in the processing the of food products.
6. *Detection of metals in pharmaceutical products:* In pharmaceutical products, the small amount of a catalyst that is used in manufacturing process may be present

in the final product. By using AAS, the amount of catalyst present in the final product can be determined. This is also used for detection of many trace elements such as zinc in multivitamins, lithium in antidepressants, etc. It can be used for detection of traces of metals in parenteral preparations.

7. *Detection of metals in environment:* AAS is widely used to monitor—e.g., it can be used to find out the levels of various elements in rivers, drinking water, sea water, air, and petrol especially for the leaching of zinc.
8. *Detection of trace elements in cosmetic products*: The content of heavy metals such as lead and cadmium used in cosmetic products can be analyzed using AAS.
9. It is also used in mining industry for the detection and determination of precious metals.
10. *AAS is a life-saving technique:* In Canada, AAS was used to determine the unsafe levels of lead in children who was lived nearby a lead smelter. In Japan, from 1932 to 1968, AAS was used to identify the reason why over 3000 residents who lived near the Minamata Bay started showing neurological problems and pregnant women started giving birth to impaired children. Scientists started taking samples and performed AAS process; AAS results showed a very high concentration of mercury in their blood. This was resulted on stopping Chisso corporation dumped about 27 tons of mercury in the bay.
11. It is also used for the analysis of gas for the purity.

6.10 Precautionary Measures

AAS is used for the analysis of many different elements. It can be potentially harmful as well

1. *Exhaust system*: A large amount of heat, fumes, and vapors are produced in AAS that may be harmful for operator. It is very important to use the exhaust system to expel the excess amount of heat, fumes, and vapors that are produced during the working of AAS.
2. *Gas cylinders*: It must be located outside of the laboratory in a cool and well-ventilated area. Proper ventilation is practiced for protection of formation of potentially hazardous toxic fumes.
3. *Flammable solvents*: The combination of solvent and flame is a harmful situation to the operator. The analysis through AAS always uses solvent with the highest flashpoint. It is recommended to use the covered containers and smallest practical volume.
4. *Burners*: It is recommended that the burners should be kept close to any door and/or material made up of wooden and/ plastic material. Before the operation, the inspection of the entire burner system is necessary.
5. *UV radiation*: Hazardous UV radiation is emitted from flames, analytical furnaces, hollow cathode lamps. Do not view the flames directly unless you are wearing protective goggles. During the working on AAS, the door or flame shield must be closed.

6.11 Advantages

This technique has following advantages:

1. *Highly sensitive*: It is highly sensitive technique for the detection of metals.
2. *High accuracy*: It is a method of high accuracy, if appropriate standards are followed.
3. *High selectivity*: It is used for the detection of single element in the sample.
4. *Wide applicability*: It is widely used in pharmaceutical analysis, elemental analysis, and water analysis.
5. *Highly specificity*: It is highly specific because element of the particular metal can absorb the specific wavelength of light.
6. This is independent from the nature of flame.
7. It is very easy to operate.
8. The sample preparation is very easy.

6.12 Disadvantages

This technique has the following disadvantages:

1. It is applicable to analysis of metals only.
2. It is time consuming technique.
3. It is more expensive technique.
4. It needs separate lamp for each element to be determined. Each sample is analyzed separately.
5. In aqueous solution, signal is predominantly affected by the anion.
6. Thermal interference may affect the overall result.
7. It has relative low precision.
8. Analysis cannot be done simultaneously.
9. Only liquid samples can be used. The solid sample for analysis is first converted into a vapor form and then analyzed.
10. It cannot be used for analysis of lighter metals such as hydrogen, oxygen, sulfur, halogens, and noble gases.

Further Reading

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Atomic Emission Spectroscopy

7

Abstract

Atomic emission spectroscopy is the oldest elemental analysis among spectroscopic techniques and still it is popular. This technique is used specifically to determine the quantity of element in the sample. In this chapter, we have explained the basic mechanism of atomic emission spectroscopy along with its instrumentation, working, applications, advantages, and some disadvantages.

Keywords

Types of emission spectra · Components of AES · Inductively coupled plasma · Interferences of AES

7.1 Introduction

Atomic emission spectroscopy is a type of atomic spectroscopy that is frequently used in order to measure the number of elements found in various samples. The atoms of analyte are excited and promoted to relatively higher energy level by providing the sufficient amount of energy. This energy is obtained from heat energy provided from the atomization sources. Afterward, the excited atoms are shifted back to lower energy levels with the emission of light energy (Fig. 7.1). Emitted energy waves are passed across monochromators/filters before detection by photomultiplier tubes.

7.2 Principle

In atomic emission, the sample is subjected to high energy thermal environment to produce the excited state which can emit light in the form of photon. As the wavelength of light emitted by atoms or ions is specific for each element present in the sample, so the measurement of this emission spectrum which is the collection

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https://doi.org/10.1007/978-981-15-1547-7_7

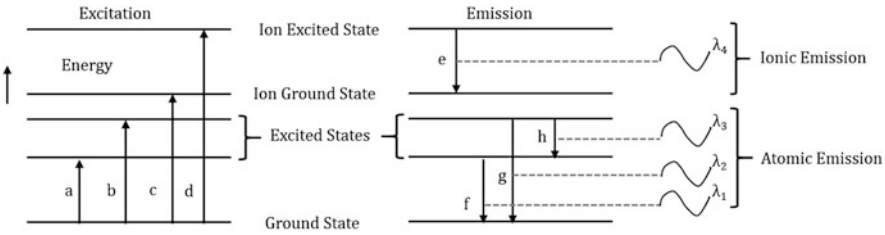

Fig. 7.1 Schematic representation of excitation and emission of energy in atomic emission spectroscopy

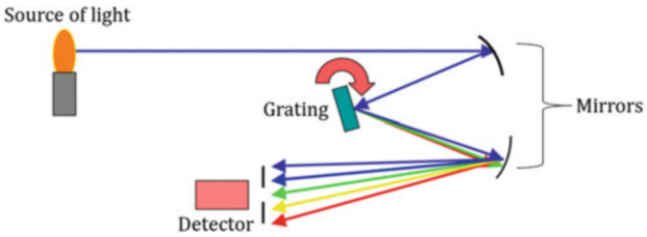

Fig. 7.2 Schematic representation of emission spectra in atomic emission spectroscopy

of emission lines from the excited atoms gives a quantitative measurement of the concentration of analyte in the sample. As the numbers of atoms or ions is increased, the amount of the emission intensity will also be increased. Every element has a unique set of energy levels and hence it will have a unique set of wavelength in which energy will be released, therefore these wavelengths are specific for every element present in the sample.

7.3 Types of Emission Spectra Used in AES

When a light beam is passed through prism/grating, it is divided into its components constituting various colors. This array of colors is known as spectrum. When an analyte is heated at high temperature by electric or thermal method, there is the emission of light. When the light, coming after passing through the prism, is analyzed with spectroscope, the spectrum so obtained is known as emission spectrum (Fig. 7.2). It has following three types:

7.3.1 Line Spectra

They consist of sharply defined and often widely and irregularly spaced individual line of the single wavelength. It is also known as atomic spectra and used for the analysis of the atoms.

7.3.2 Band Spectra

They consist of group of lines. It is also known as molecular spectra.

7.3.3 Continuous Spectra

They are obtained when solids are heated to incandescence. They are characterized by the absence of any sharp lines as a function of wavelength.

7.4 Components of AES

It has following components including sample containing analyte, source of energy to excite the atoms or ions in sample, a monochromator, a detector, and a readout device/computer (Fig. 7.3).

7.4.1 Emission Source

Plasma, arcs, sparks, and flames are the most commonly used emission sources. Plasma is a homogeneous mixture of gaseous electrons, ions, and atoms at very high temperature. There are two types of plasma most frequently used as atomic emission source, namely inductively coupled plasma (ICP) and direct current plasma (DCP).

7.4.1.1 Inductively Coupled Plasma

ICP is comprised of three concentric quartz tubes. Argon gas stream flows through these tubes at a rate of 5–20 L/min. Outer tube has diameter of approximately 2.5 cm and topmost of the outer tube is encircled by a radiofrequency powered induction coil generating power of almost 2 KW with frequency of approximately 27–41 MHz. The outer coil also generates a very strong magnetic field. A spark is used to ionize the argon and this ionized argon then interacts with the strong magnetic field and

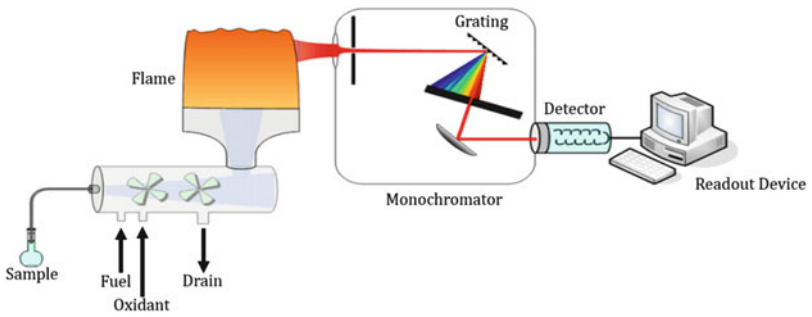

Fig. 7.3 Schematic representation of components of atomic emission spectroscopy

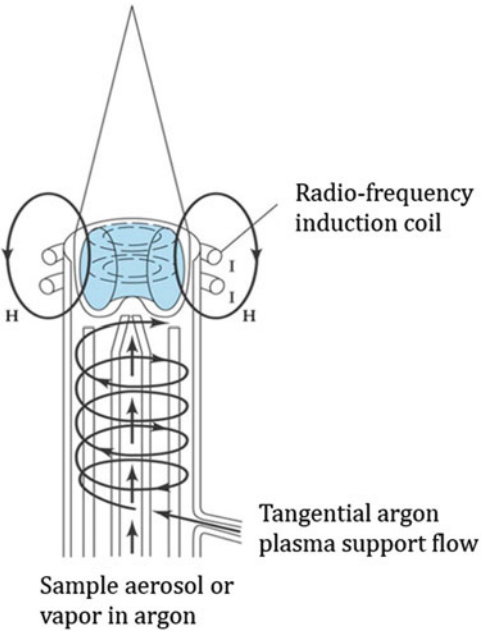

Fig. 7.4 Schematic representation of inductively coupled plasma as an emission source in atomic emission spectroscopy

forces to move in the surroundings of induction coil with a high speed. Because of this a high temperature is achieved due to high resistance because of circulating argon. The topmost of quartz tube gets a very high temperature and therefore, it should be isolated, then cooled. It may be achieved if argon is passed tangentially around the tube walls. An ICP is usually known as torch plasma. The torch is produced due to the emission of argon at a very high temperature of the plasma (Fig. 7.4).

7.4.2 Monochromator

Similar to an AAS, the monochromator acts as a wavelength selector and it separates different wavelengths and selects the required wavelength. They may be prism monochromator or grating monochromator.

7.4.3 Detector

The required wavelength from the monochromator is allowed to pass through a detector which is used to convert light signal into electrical signal. These are the devices that are responsible to detect the wavelengths which are being absorbed by the sample. The most commonly used detector in AES is photomultiplier tube which is actually vacuum photo tube and possess high sensitivity.

7.4.4 Readout Device

It is used to display the absorption spectrum and absorbance at a specific wavelength. Nowadays, the read out devices have microprocessor-controlled electronics that deliver outputs compatible with computers and printers. Thus, it minimizes the risk of operator error while transferring data.

7.5 Working of AES

Instrumentation of atomic emission spectroscopy is almost the same as that of atomic spectroscopy with a slight difference. Light source is not required. Atomization source is most important component here which is required for the atomization of sample and excitation of free atoms. When atoms reached in their excited state they move to the ground state with the emission of light which passes through the monochromator which isolates the specific wavelength for a specific element. This specific wavelength then enters in detector that converts light signal into electrical signal. At the end, the absorption spectrum is displayed on the screen of the readout device.

7.6 Comparison Between AAS and AES

Comparison between AAS and AES has been described in Table 7.1.

7.7 Interferences of AES

1. *Ionization Interference:* During high flame temperature, metal such as potassium may completely lose an electron that ultimately reduces the observed emission from the sample.
2. *Viscosity interference*: Organic substances may either decrease or increase the rate with which it draws towards flame compared to a standard solution. For example, sucrose usually decreases the rate, consequently giving false negative

Table 7.1 Comparison of atomic absorption spectroscopy with atomic emission spectroscopy

AAS	AES
It depends upon the number of atoms present in ground state	It depends upon the number of atoms present in excited state
It measures the radiations absorbed by the atoms in ground state	It measures the radiations emitted from atoms in excited state
Light source is present	Light source is absent
The temperature of the atomizer is adjusted for the atomization of atoms of analyte present in the ground state only	The temperature of the atomizer is high enough in order to atomize the atoms of analyte and excite them to a greater energy level

results, on the other hand, ethanol may increase the rate, consequently giving a false positive result.
3. *Anionic interference*: Anions like sulfates and phosphates form non-volatile salts by reacting with metal ions and decrease the reading of a given sample solution. The anions may be removed with the addition of lanthanum chloride, it participates them out and replaces them with the chloride anions.

7.8 Applications of AAS

7.8.1 Analysis of Pharmaceuticals

1. For quantitative analysis of alkali metals in alkali metal salts, dialysis solutions, and infusions.
2. For determination of metallic impurities in some inorganic salts that are used in the preparation of many solutions.
3. To detect lithium, sodium, and potassium in some raw materials of pharmaceuticals.
4. To detect metals during IPQC and final dosage forms of pharmaceuticals.

7.8.2 Biomedical Applications

1. Detection of metals in biological fluids.
2. Detection of metals (Cu) in brain.
3. Estimation of sodium salts in breast milk.

7.9 Advantages

This technique has the following advantages:

1. High accuracy.
2. High resolution.
3. Low stray light.
4. Wide dynamic range.
5. High precision.
6. Highly reproducible.
7. Preferred over atomic absorption spectroscopy as all the atoms in a sample are detected at the same time.

7.10 Disadvantages

This technique has the following disadvantages:

1. Expensive.
2. Destroying the sample.
3. Used mainly for metals.

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Molecular Emission Spectroscopy

8

Abstract

In this chapter, the steps involved in molecular emission spectroscopy have been completely described. The types of fluorescence are schematically discussed. There are multiple components of molecular emission spectroscopy that are needed for proper functioning. The factors that affect the fluorescence have been described along with quenching. At the end of chapter, application of AES has also been discussed.

Keywords

Types of luminescence · Types of fluorescence · Fluorescence intensity · Quenching

8.1 Introduction

Molecular emission spectroscopy is an analytical technique that is used for measurement of the emission from excited atoms of elements present in samples by quantitative. The energy to excited atoms is provided by the radiation source. It is the phenomena in which the molecules of element present in sample solutions absorb the radiation of specific wavelength (at ground state having low energy level) and get excited (at excited state having low energy level). At excited state, the molecules are unstable and emit the radiation. The emitted radiation is estimated from the excited molecules in the sample after the absorption of radiations of specific wavelength which is referred as excitation wavelength. The measurement of the emitted radiation at a longer wavelength is known as fluorescence or emission wavelength. Molecular emission spectroscopy is a type of spectroscopy in which the atoms and/or molecules during the transition from an excited state to ground state, emit the radiation of specific wavelength which can be measured (Fig. 8.1).

What happens after a molecule has absorbed light? (Fig. 8.2). A large number of substances are known which can absorb UV or VIS light energy and lose excess

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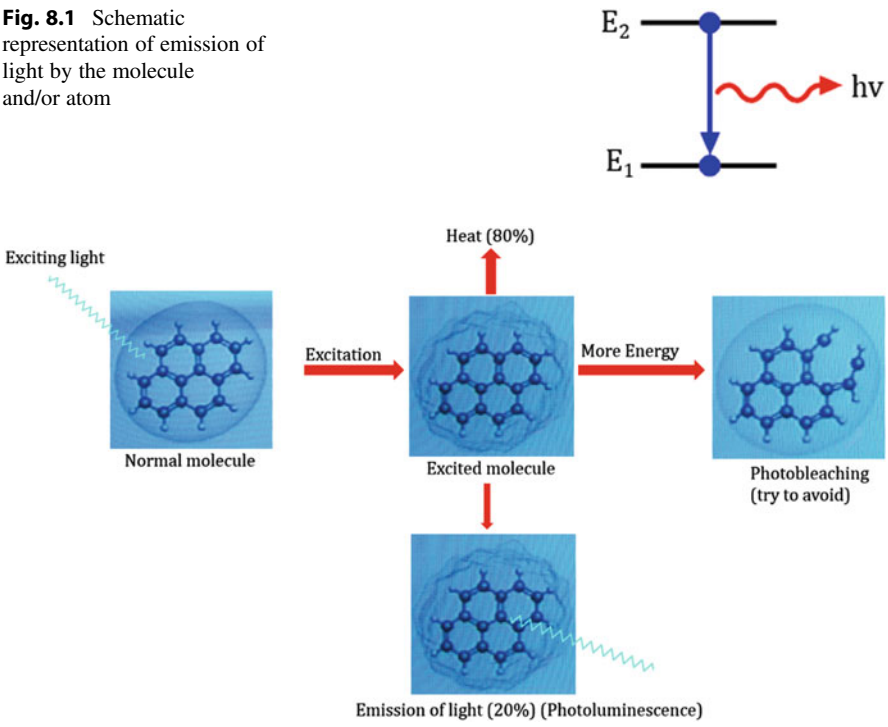

Fig. 8.1 Schematic representation of emission of light by the molecule and/or atom

Fig. 8.2 Schematic representation of absorption of light by molecule

energy as heat through collision with neighboring atoms or molecules. But some substances also lose only part of this excess energy as heat and emit the remaining energy as ER wavelength longer than that absorbed. This process of emitting radiation is known as luminescence or photoluminescence and the substance which exhibits such characteristics is known as luminescent.

8.2 Types of Luminescence

8.2.1 Fluorescence

When a substance absorbs radiation and immediately emits the radiation after the radiation absorption, this phenomenon is called as fluorescence. The substances which exhibit phenomenon of fluorescence are called as fluorescent. The phenomenon of fluorescence happens instantaneously and begins immediately after the absorption of radiation of light and terminates quickly as the incident light radiations are cut off.

8.2.2 Phosphorescence

When a substance absorbs the radiation and subsequently emits the radiation continuously after the absorption of radiation is cut off. This type of phenomenon is called as phosphorescence. The substances exhibit such characteristic are called as phosphorescent substances.

8.2.3 Chemiluminescence

During the process of chemiluminescence, light is produced from chemical reaction. During chemical reaction, the light is emitted because this reaction produces electrically excited molecules. When these excited molecules return to their ground state, the emit light. In many biological systems, the chemiluminescence reactions occur where this phenomenon is known as bioluminescence. One of the distinguished characters of chemiluminescence is that it does not need any sophisticated instrumentation. Additionally, no external radiation source is also required for excitation purpose. The instrument may contain only two things, one is reaction vessel and the other one is a photomultiplier tube. The devices that are used for the selection of wavelength are not needed because the source of radiation is only chemical reaction. Moreover, there is no need of excitation source. Chemical reaction delivers the energy in order to excite the molecules. In its simplest form, chemical reaction can be represented as follows:

$$\mathrm{A} + \mathrm{B} \rightarrow \mathrm{C}* \rightarrow \mathrm{C} + \mathrm{h}\nu.$$

Chemiluminescence has wide range of applications. It can be used for the detection of arsenic in water and can also be applied to fabricated microarrays on a flow chip, allowing for patterned biosensor applications.

8.3 Theory

Following steps (Fig. 8.3) are involved in molecular emission spectroscopy:

1. *Vibrational relaxation:* There is a transfer of surplus energy from vibrationally excited specie to the solvent molecules. This process happens within very short time span of approximately 10^{-15} s and solvent molecules return back in the low vibrational energy state from an electronically excited state. The molecules that are found in the singlet excited lose the energy comparatively easier due to the collision with surrounding molecules of solvent.
2. *Internal conversion:* When the lower and upper electronic states of the excited singlets have same multiplicity, this phenomenon is known as internal conversion.

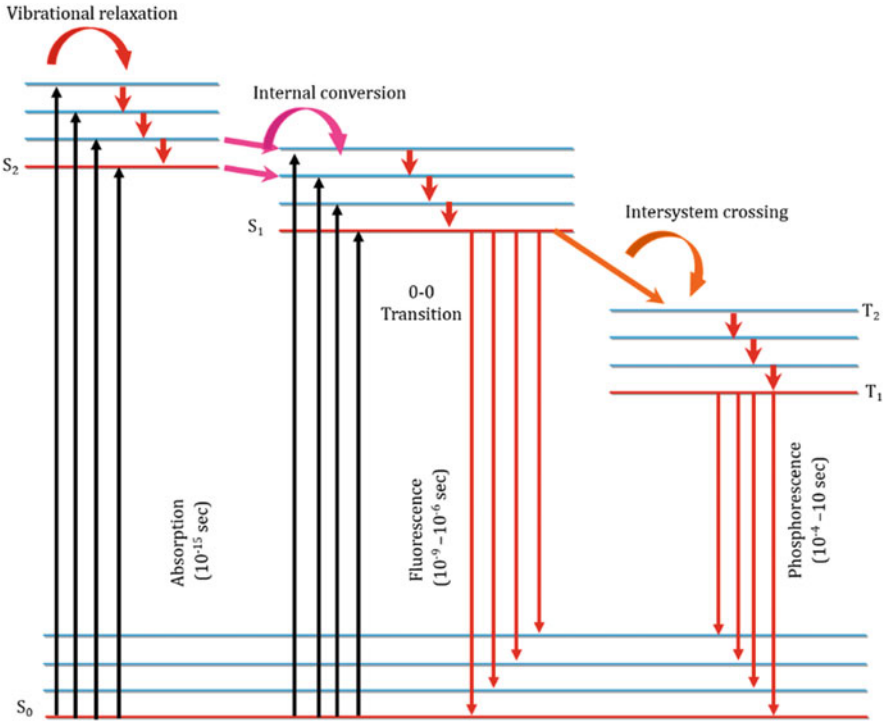

Fig. 8.3 Schematic representation of electronic levels and transitions in a fluorescence and phosphorescence

3. *Photon emission*: The molecules in the singlet excited state come back to the ground state and cause the emission of the photon that is called as fluorescence.
4. *Energy transfer*: The energy is transferred from singlet state to the triplet state when the molecule come back to the ground state which is also called as intersystem crossing.

Figure 8.4 briefly describes how fluorescence excitation-emission cycle takes place on emission spectroscopy.

8.4 Principle

When one electron from an electron pair present in a molecule is get excited (Fig. 8.5) to a high level of energy then may be a singlet or triplet state produced. During the excited singlet state of molecule, the spinning movement of the excited electron remains opposite to that of the left-over ground state electron. However, during the triplet state, the spinning movement of both electrons becomes parallel

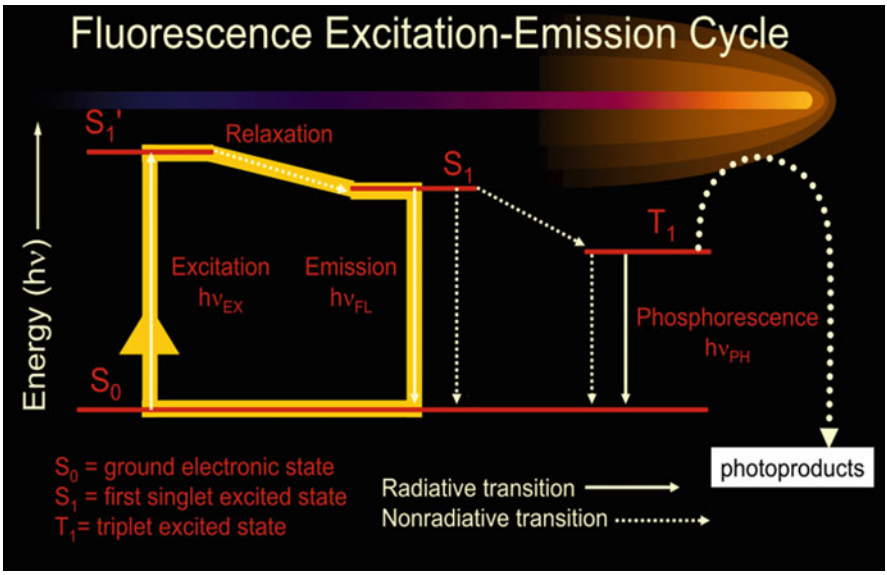

Fig. 8.4 Schematic representation of fluorescence excitation-emission cycle

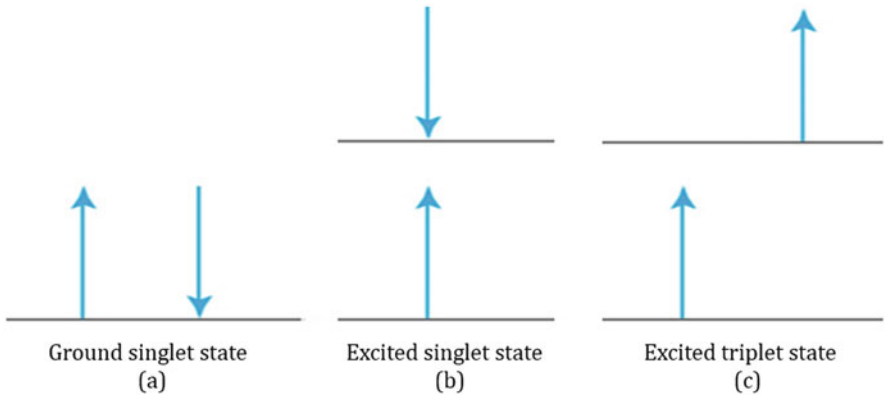

Fig. 8.5 Schematic representation of states of the electrons in molecule in ground and excited states

and unpaired. The excited singlet state is comparatively more energetic as compared to its corresponding unpaired singlet state.

8.5 Types of Fluorescence

Following are the important types of fluorescence as shown in Fig. 8.6:

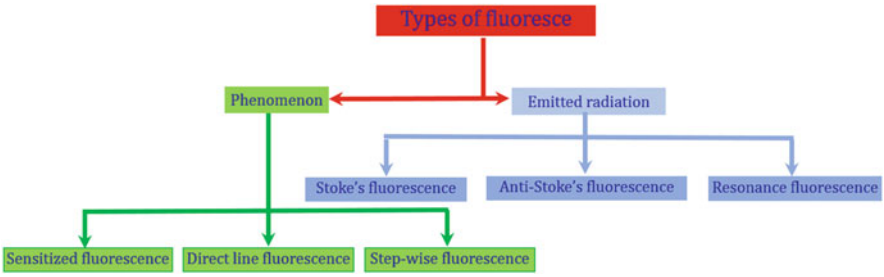

Fig. 8.6 Schematic representation of types of fluorescence

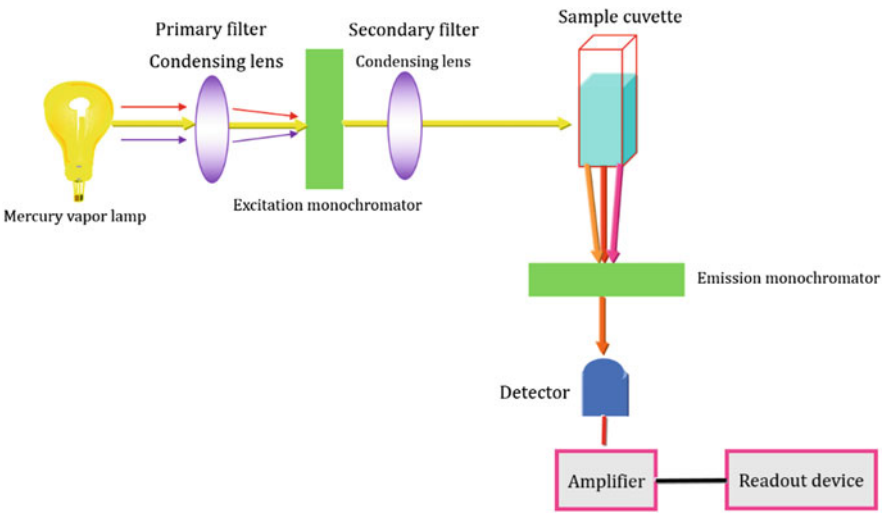

Fig. 8.7 Schematic representation of components of atomic emission spectrophotometer

8.6 Instrumentation

Following are the important components of AES which have been schematically illustrated in Fig. 8.7:

8.6.1 Radiation Source

The plasma can be generated by applying the potential difference between the two electrodes. The generation of plasma by this way is known as direct current plasma. For this purpose, plasma supported gas is needed. An argon is commonly used for this purpose. In spectroscopic analysis, for desolvation and vaporization of sample, heat may also be provided by the flame.

8.6.2 Filters

Monochromator or filter is a very essential part in an atomic emission spectrometer. It is used for the separation of all the thousands of lines. If good monochromator is not used, the efficiency of spectrometer is severely compromised.

8.6.3 Detectors

In atomic emission spectroscopy, the most commonly used detector is photomultiplier tube (PMT). PMT is used for the detection of weak signals. In photomultiplier tube, photons are absorbed which result in the emission of electrons. PMT is highly sensitive for UV, VIS, and near-IR regions of EMR.

8.6.4 Amplifiers

It amplifies the signals received from the detector and then transfer to the read-out device.

8.6.5 Read-out Device

It is used to display the spectrum. Read-out devices work in conjunction with multiprocessor-controlled electronics that deliver the output to computer and printer. It minimizes the risk error by operator. The most widely used read-out devices are:

1. Digital voltmeter.
2. Simple galvanometer.
3. Potentiometer.
4. Computer.

8.7 Factors Affecting the Fluorescence Intensity

Following are the most important factors that commonly influence the working of AES:

1. Temperature
2. Viscosity
3. Nature of solvent
4. pH
5. Presence of solutes
6. Presence of functional groups
7. Nature of the molecule

8. Structure of the molecule
9. Light

8.8 Quenching

The decrease in fluorescence intensity is known as quenching.

8.8.1 Reasons of Quenching

1. Concentration of the molecule
2. pH
3. Presence of chemical substances
4. Temperature
5. Viscosity

8.8.2 Types of Quenching

1. Self-quenching
2. Collisional quenching
3. Static quenching
4. Chemical quenching

8.9 Applications

1. Used for the determination of inorganic ions.
2. Widely used in the field of pharmaceuticals.
3. Estimation of metals in biological fluids.
4. Estimation of vitamins.

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Mass Spectrometry

9

Abstract

In this chapter, we have described the instrumentation of mass spectrometer along with comprehensive description of sources of ionization mechanism of ionization and types of analyzers. Moreover, the description of peaks in the mass spectrum has been given in this chapter. At the end of this chapter, application along with advantage and disadvantages has been given.

Keywords

Electron ionization · Chemical ionization · Atmospheric pressure ionization · MALDI · Mechanism of ionization

9.1 Introduction

A mass spectrometer is a device that determines the weight of the molecules by estimating the mass to charge ratio (m/e) of ions. In this technique, molecules are bombarded with electrons; high energized positive ions are produced which are further breakdown to produce small fragments. These ions are separated in magnetic or electric field according to their mass to charge ratio. Figure 9.1 illustrates how mass spectrometer works.

This technique is used for studying the effect of ionization energy on the molecules. It depends upon which type of chemical reaction takes place in the sample which exist in the form of gaseous phase. The function of mass spectrometer is associated with its components. Such as ion source is responsible for the production of ions. The mass analyzer separates the ions on the basis of their mass to charge ratio. Detector measures the separated ions and the results are displayed on mass spectrum. The mass spectrum is plotted between ions abundance and mass to charge ratio. This is used for the detection of isotopes on the basis of their mass. Nowadays, it is used in combination with the gas chromatograph for the estimation of quantities of the contaminants and toxins.

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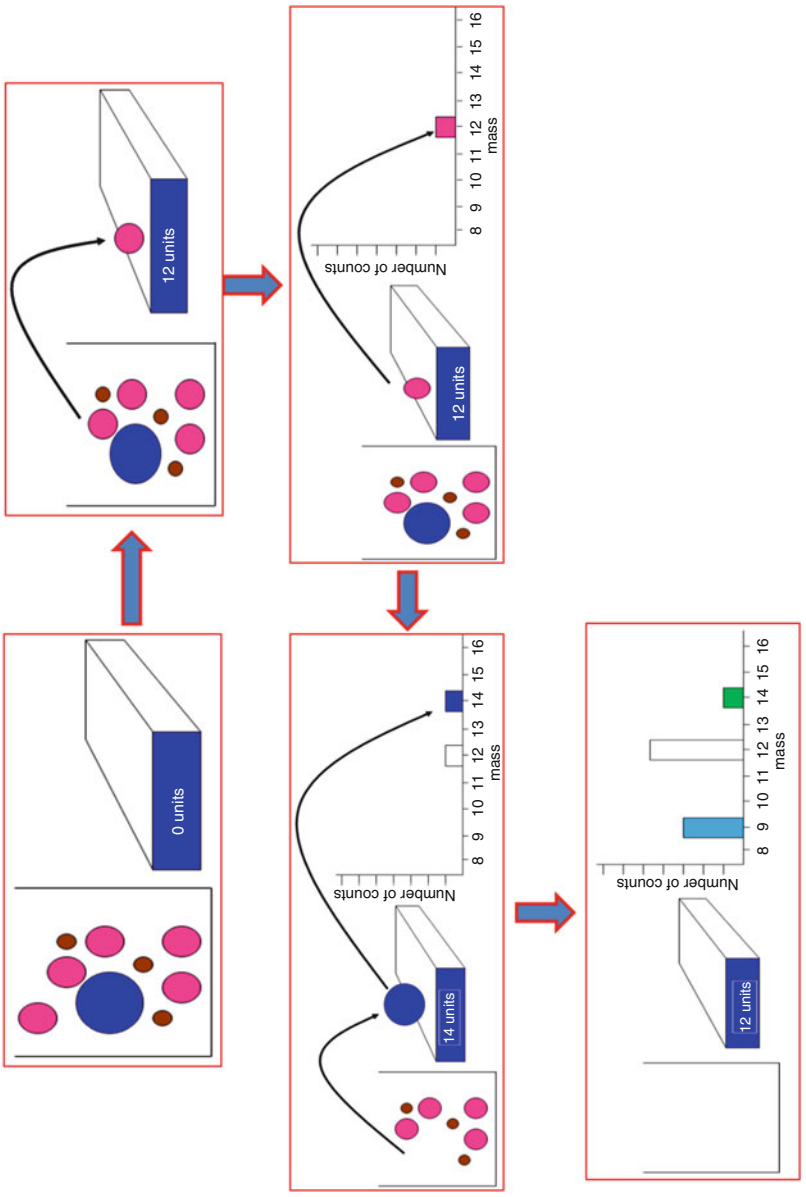

Fig. 9.1 Schematic representation of how mass spectrophotometer works?

9.2 Principle

When compound is bombarded with an electron, the compound has tendency to lose one electron and form the metastable ions as represented in the following equation:

$$M \xrightarrow{e^-} M^+ + e^-.$$

The increase in energy will lead to the production of cations which are determined by the mass spectrometer which is based on their m/e of positive ions. Mass to charge ratio (m/e) is the mass of samples divided by the charge of the sample. Ions give information about the structure and nature of the sample molecule. The ions which has high mass to charge ratio, they are heavier isotopes and vice versa. The molecular mass of these separated ions is also determined. The separated ions on the basis of m/e ratio are determined in proportion to their abundance. The result will be obtained in the form of mass spectrum. The mass spectrum is plotted between the ion's abundance and m/e ratio. By this way, a mass spectrum of the molecule is thus produced. It displays the result in the form of a plot of ion abundance versus m/e ratio.

9.3 Instrumentation

Following are the main components of mass spectrometer. A schematic representation of components of mass spectrometry has been illustrated in Fig. 9.2:

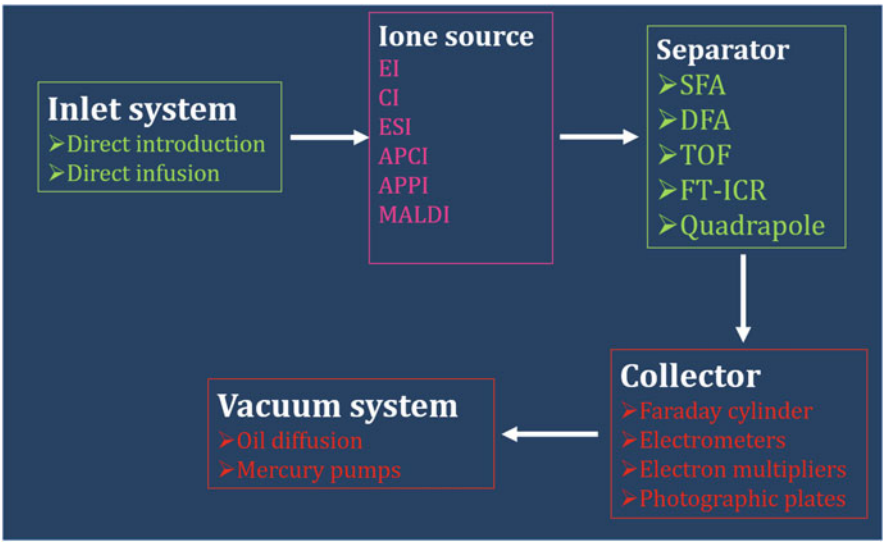

Fig. 9.2 Schematic representation of components of mass spectrophotometer

9.3.1 The Inlet System

The sample is placed in mass spectrometer at an atmospheric pressure. There are two ways by which the sample can be introduced into the mass spectrometer.

9.3.1.1 Direct Introduction

Initially, the sample is introduced in the probe of mass spectrometer and then placed into the ionization source.

9.3.1.2 Direct Infusion

The sample capillary is used to introduce the sample into the ionization source.

9.3.2 The Ion Sources

It is a mechanical device which is used to convert the sample into ions. It is process of charging a molecule. In mass spectrometry, generally, three types of samples are used for the analysis of solid, liquid, and/or gas samples. Following methods are used to charge the molecules and its schematic representation has been illustrated in Fig. 9.3:

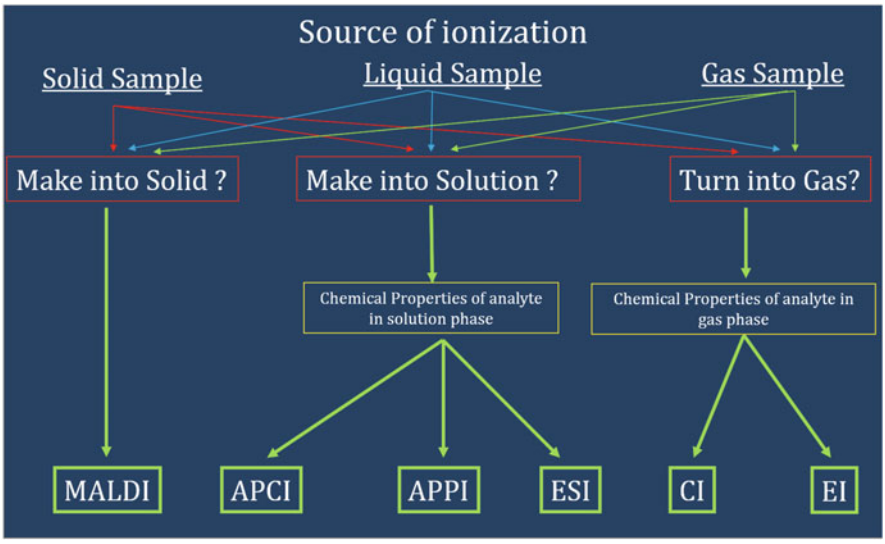

Fig. 9.3 Schematic representation of sources of ionization. *CI* chemical ionization, *EI* electrical ionization, *ESI* electrospray ionization, *APPI* atmospheric pressure photo-ionization, *APCI* atmospheric pressure chemical ionization, *MALDI* matrix-assisted laser desorption ionization

9.3.2.1 Electron Ionization

It is the most commonly used ionization process that is used in mass spectrometry. It can be used for variety of gaseous phase molecules. It is mostly used for spectral libraries. It produces the large number of fragments that is why all the molecular ions are not observed. Fragmentation is responsible for providing the information about the structure by interpreting the unknown spectra. For the purpose of ionization, the electrons are required. These electrons can be produced by passing the current through a filament. The number of electrons produced by filament is controlled by the amount of current. These electrons move due to electric field present in the source region. As a result, a high energy electrons beam is produced. When this high energy beam passes through a sample, ions are formed because electrons are removed from the sample. After the ionization of molecules, the spectrum is produced. The spectrum shows the separation of the product ions. The ions which have less molecular energy act as intact molecular ions. The molecular ion that has high energy undergoes the process of fragmentation. The group of these resulting fragments is known as product ion. These product ions can be determined by ionization energy and kinetics of fragmentation pathway. The change in kinetic energy causes the distribution of different fragment ions. With the help of distribution, information can be interpreted in the form of mass spectra.

9.3.2.2 Chemical Ionization

In this process, ionization of analyte in gaseous state happens by a gaseous phase ion molecule reaction instead of the field ionization by direct electron impact and photon impact. The electron ionization of gas is usually occurred due to ion molecule reaction. Due to this reaction, ions are formed. The gas which is present in excess amount is neutral for the production of chemical ionization. The collision of these reagent ions with analyte to produce ionic character in the analyte. Then, analyte which is formed initially passes through the fragmentation process and sometimes these may react further with reagent gas for the formation of array of ions. These arrays of ions produce the mass spectrum of analyte by using specific gas.

9.3.2.3 Electrospray Ionization

Electrospray ionization is a very soft ionization method that is particularly used for the determination of molecular weight of peptides, proteins, and other biological fluids. This technique is very helpful especially when we analyzing the sample having high molecular weight. Fragmentation of sample into small charged particle is not involved in this technique. In this process, the macromolecules are converted into small droplets by ionization. These small droplets undergo desolvation process and very small droplets are produced. These very small droplets produce molecules having the attached protons. These molecular ions after the protonation and desolvation process pass through the mass analyzer and then to detector. The mass of the interested sample can be estimated by using this technique.

9.3.2.4 Atmospheric Pressure Ionization

The source of atmospheric pressure ionization (API) can ionize the sample at atmospheric pressure and these ions are then transferred to the mass spectrometer. API is used for temperature senstive samples such as polymers, proteins, and peptides. By dissolving the sample into an appropriate solvent, a solution is formed. This newly formed solution is placed into mass spectrometer. Due to conventional inlet, solvent produces more pressure in the source zone of mass spectrometer. When the liquid enters into a vacuum, the solent freezes due to Joule Thomson effect. This reduces the efficiency due to the formation of cluster. To avoid this problem, analyte must pass through a series of pumps. In this process, solvent passes through the nozzle which converts solvent into small droplets and also get charged. The charge density of droplet increases due to evaporation of solevent that caused the shrinkage of droplet and charge density increase on the droplets. These droplets eventually reached at a point where the surface tension that is responsible for holding these droplets is less than repulsion from an electric charge. Aa a result, droplet explodes to produce the multiple ions. The spectra will represent the molecular ions having the different charge numbers.

9.3.2.5 Atmospheric Pressure Photo-Ionization

A way in which ionization is done by photo-ionization. In this process, a dopant absorbs the light then it is added to a sample for the ionization of the sample. The estimation of small molecules in biological mixture becomes very crucial part for the drug discovery.

9.3.2.6 Atmospheric Pressure Chemical Ionization

This method uses the source of electrospray ionization. But voltage is applied on a needle that produces corona charge at atmospheric pressure. Due to this discharge, the ions are produced such as hydronium ion or water cluster. The sample is discharged through the spray which is produced when heated gas is mixed with liquid and as a result sample is evaporated. Transfer of the proton from water cluster or hydronium ion causes the production of ions. These ions are passed through the opening of vacuum.

9.3.2.7 Matrix-Assisted Laser Desorption Ionization

Matrix-assisted laser desorption ionization (MALDI) is based on the ionization which can be done by the bombardment of sample molecules along a laser light for the ionization of sample. MALDI is a soft ionization technique in which a laser strikes with a matrix of many small molecules in order to make a gaseous state from the sample molecule without causing the fragmentation or decomposition in sample. Some high molecular weight biomolecules can be decomposed by heating and some conventional techniques can also cause the fragmentation or destruction of macromolecules. MALDI is suitable to analyze the biomolecules like saccharides, lipids, peptides, or many other organic macromolecules. The sample is mixed with a very high absorbing matrix compound (X) and then dried on a plate. An analyte is embedded in a very large amount of the matrix compound that is deposited on a solid

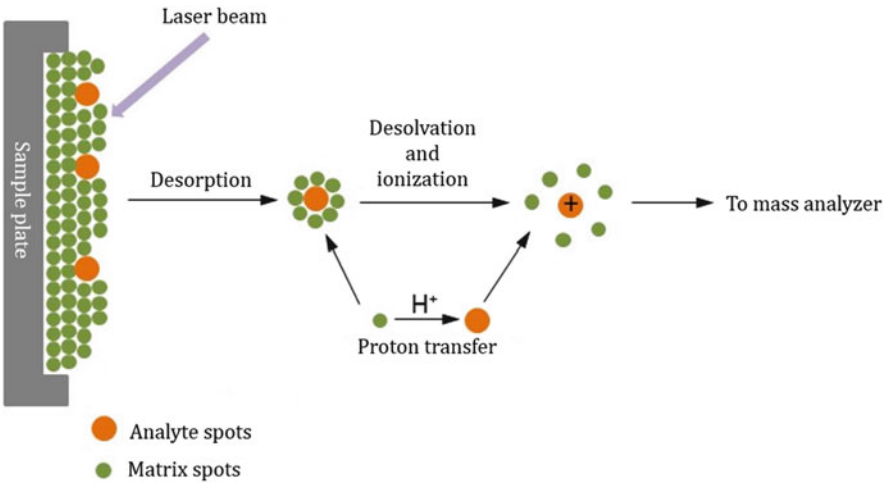

Fig. 9.4 Schematic representation of mode of action of MALDI in mass spectrometry

surface known as target. The target is constructed of a conducting metal and having many spots. After a very brief laser pulse, the irradiated spot excited vibrationally due to heating. The matrix molecules energetically excited from the sample surface and absorbed the laser energy, as a result the analyte molecules are converted into the gaseous phase. During the excitation process, the analyte molecules are getting ionized due to protonation or deprotonation with the nearby matrix molecules. A schematic representation of mode of action of MALDI has been illustrated in Fig. 9.4.

9.3.2.8 Mechanism of Ionization

Protonation

When a proton is added to a molecule then it increases the positive charge.

$$M + H^+ \rightarrow MH^+.$$

It is used in MALDI, electrospray ionization and, APCI. The samples used are carbohydrates.

Deprotonation

A proton is removed from a molecule which causes production of cations.

$$M - H^+ \rightarrow (M - H)^+.$$

It can be used in MALDI, APCI, and electrospray ionization. The sample used in this process is salicylic acid.

Cationization

This can be done by the addition of cation into the molecule along with an ammonium or alkali. This is a very stable method as compared to that of protonation.

$$\mathrm{M} + \text{cation} \rightarrow \mathrm{Mcation}^{+}.$$

It is used in MALDI, APCI, and electrospray ionization. The sample used is D-galactose.

Charge Transfer

This is also called as desorption. In this method the sample solution is converted into gas state. It is particularly used for charged complexes. This method cannot be used for many other compounds.

$$\mathrm{M}^{+}(\text{solution}) \rightarrow \mathrm{M}^{+}(\text{gas}).$$

It is used in MALDI and electrospray ionization. The sample used is tetraphenyl phosphine.

Electron Ejection

This can be done by removing the electron from molecule to form the positively charged molecule. It is used in the process of electron ionization. The sample used is anthracene.

$$\mathrm{M} \overset{-\mathrm{e}^{-}}{\rightarrow} \mathrm{M}^{+}.$$

Electron Capture

Addition of electrons to the sample molecule by absorption or capture.

$$\mathrm{M} \overset{+\mathrm{e}^{-}}{\rightarrow} \mathrm{M}^{-}.$$

9.3.3 Electrostatic System

The cations produced from the ionization source are allowed to pass through the electric field. Electric field is produced between the repeller plate and the accelerator plate which causes the acceleration of the ions of masses m_1, m_2, and m_3 to their final velocities.

$$\text{Energy } e\mathrm{V} = \tfrac{1}{2}m_1{v_1}^2 = \tfrac{1}{2}m_2{v_2}^2 = \tfrac{1}{2}m_3{v_3}^2.$$

9.3.4 Ion Separator

This is commonly called as analyzer in which sample molecules are separated on the basis of their masses.

9.3.4.1 Types of Analyzers

Single Focusing Analyzer (FSA)
At an applied voltage, all the ions which are ionized have the same energy. In this instrument, single magnet sector is used to generate the magnetic field. The ions are separated on the basis of mass to charge ratio values by the generation of magnetic field. It consists of horse shaped glass tube, small inlet, source for bombarding the electrons and accelerated plates on one end and collector slit on another end. The curvature tube provides electric and magnetic field. The analyte in the vaporize form allows to pass through the inlet and bombarded with electrons. As a result, one electron escapes from each molecule and consequently becomes a positive charge. By getting charge, they are accelerated by plates and moves in a straight path. Due to electric and magnetic field, the ions are separated on the basis of charge to mass ration. The mass spectrum is obtained by these fragments.

Double Focusing Analyzer (DFA)
It is specifically used for achieving the high resolution. Two ion beams are allowed to pass through and at the end, these are detected by two separate collectors. In this instrument, an electric sector is interposed to provide the double focusing analyzer. This has high resolution than single focusing analyzer. It is used to differentiate the small differences in the segment.

Time of Flight Analyzer (TOF)
By changing their direction, the ions can be separated. These separated ions travel through an air-free zone, the time utilized by these ions to travel this distance is known as the flight tube. It has poor mass resolution. Heavier ions move slowly than the lighter ones. A schematic illustration of TOF has been represented in Fig. 9.5.

MALDI is based on the bombardment of sample molecules with a laser light to bring about sample ionization. The sample is pre-mixed with a highly absorbing matrix compound. The matrix transforms the laser energy into excitation energy for the sample, which leads to sputtering of analyte and matrix ions from the surface of the mixture. In MALDI-TOF mass spectrometry, the ion source is MALDI and the mass analyzer is time of flight (TOF) analyzer (Fig. 9.6).

Quadrupole Analyzer
Quadrant of four parallel circular tungsten rods filter the ions by focusing on the ions through an oscillation with the help of radiofrequency. Quadrupole mass analyzers are constructed of four rods with a hyperbolic or circular cross section (Fig. 9.7). Each pair of opposing rods has positively or negatively charged. The ions in the analyzer are separated on the basis of mass to charge ratio of ions which depend

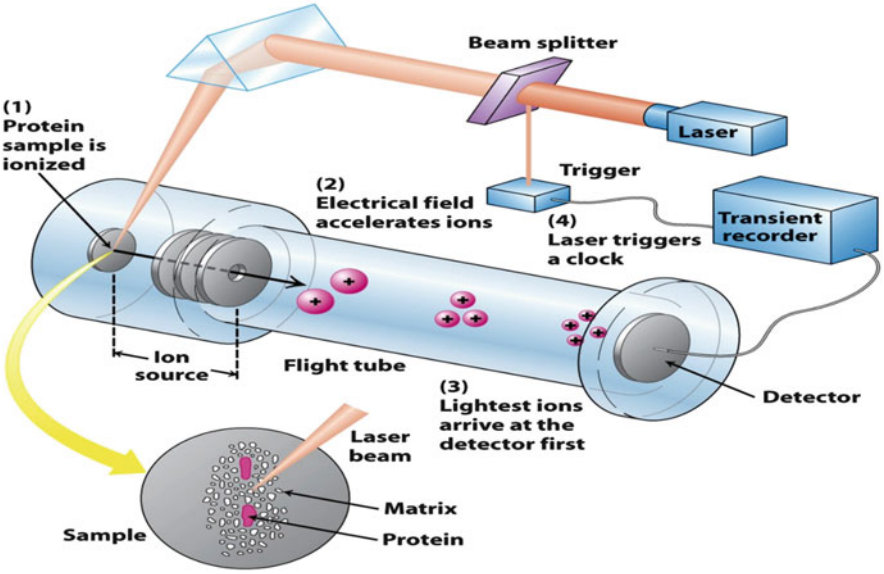

Fig. 9.5 Schematic representation of time of flight (TOF) analyzer

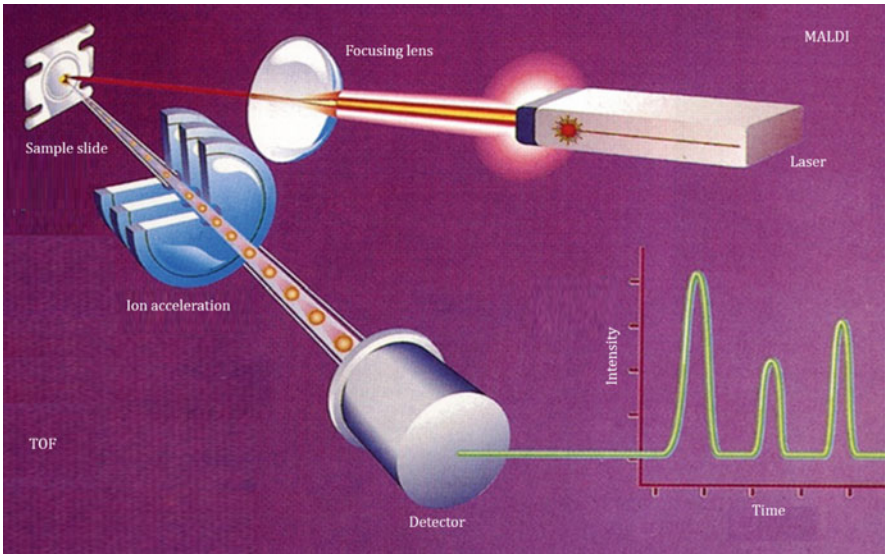

Fig. 9.6 Schematic representation of MALDI-TOF

upon their trajectories when these ions are exposed to the electric field in the space present between the rods. Oscillate ions travel in an applied electric field (the quadrupole field) between the paired rods of the quadrupole. By altering the

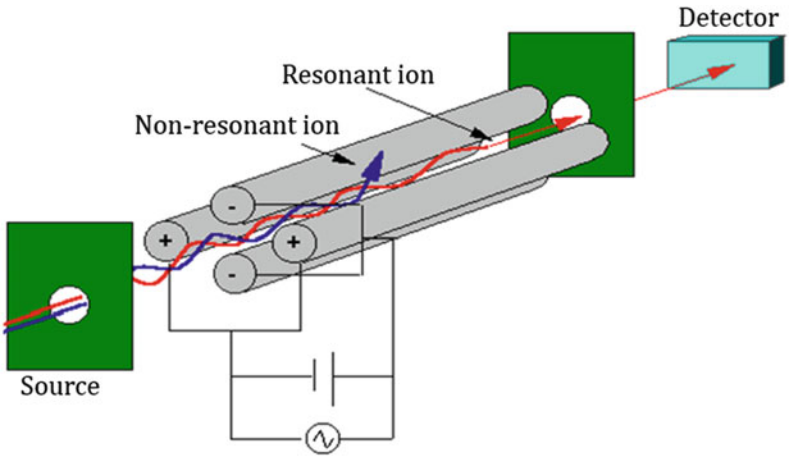

Fig. 9.7 Schematic representation of quadrupole analyzer in mass spectroscopy

characteristics of the field, manipulation of ions and molecules having the specific m/z ratio (molecule A^+) will start to oscillate with a harmonic ion trajectory by creating an ion beam that navigates the quadrupole. All other ions (molecule B^+) can be filtered out of the ion beam.

Fourier Transform Ion-Cyclotron Resonance (FT-ICR)
It is also called as penning ion trap in which magnetic field is used to trap and store the ions.

9.3.5 Ion Collector

It is also known as ion receiver. Following are the types of ion collector:

1. Photographic plates.
2. Electron multipliers.
3. Electrometers.
4. Faraday cylinders.

9.3.6 Vacuum System

In this system, mercury and oil diffusion pumps are most commonly used.

- Inlet vacuum = 0.015 Torr.
- Ion source = 10^{-5} Torr.
- Analyzer = 10^{-7} Torr.

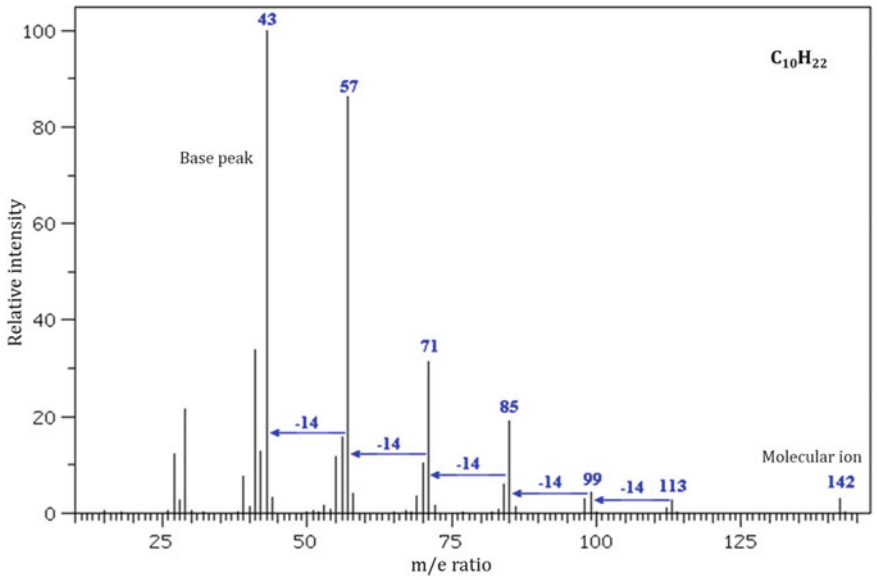

Fig. 9.8 Schematic representation of types of peaks in mass spectroscopy

9.4 Types of Peaks

There are several types of peaks that can be observed during analysis (Fig. 9.8). The types of these peaks have been described as follows:

9.4.1 Molecular Peak

It is also called as parent peak. This peak can be produced by the bombardment of the sample to lose one electron and then this peak can be obtained.

9.4.2 Fragment Peak

This peak is observed when the fragment ions produce when energy is supplied to the molecular ions.

9.4.3 Rearrangement Ion Peak

This peak is obtained due to the rearrangement of the fragment ions.

9.4.4 Metastable Ion Peak

The ion produced from the source and analyzer is called as metastable ion and the peak which is obtained is called as metastable ion peak. These obtained peaks are broader having the low intensity.

9.4.5 Multicharged Ion Peak

The peaks produced from ions which may have more than one charge are called as multicharged ion peaks, e.g., CO_2, N_2, CO.

9.4.6 Base Peak

The largest peak in the spectrum is called as base peak.

9.4.7 Negative Ion Peak

After the increase of energy positive ions are formed and the negative ions also exhibit the peaks, but these peaks are negligible in MS.

9.5 Types of Mass Spectrometry

Following are the most important types of mass spectrometry:

9.5.1 Gas Chromatography-Mass Spectrometry (GC-MS)

When GC is combined with MS, it increases the sensitivity of the identification and structure elucidation of the compounds. GC separates the semi-volatile and volatile compounds, but it does not identify these compounds whereas, MS does this job.

9.5.1.1 Modes of Operation of GC-MS

Following three modes are used:

1. Spectral mode.
2. Total ion current mode.
3. Selective ion monitoring mode.

9.5.1.2 Advantages

1. Highly sensitive.
2. Powerful instrument for qualitative and quantitative analysis.

9.5.1.3 Disadvantages

1. Time consuming.
2. Non-volatile compounds cannot be used.
3. Thermolabile compounds cannot be used.

9.5.2 Liquid Chromatography-Mass Spectrometry (LC-MS)

This is the combination of LC and MS.

9.5.2.1 Advantages of LC-MS

1. Most advanced technique than GC-MS because no heating is required.
2. Non-volatile and thermolabile can be used for analysis.
3. Mainly used for structural and molecular weight determinations.
4. Retention time is less than that of GC-MS.
5. Highly sensitive than that of GC-MS.
6. Operating is comparatively easy than that of GC-MS.

9.5.3 Chemical Ionization Mass Spectrometry (CI-MS)

CI-MS is commonly used for the physicochemical characterization of the molecules such as ions strike with the molecules which are present in the ion source. Chemical ionization is the basic phenomenon for CI-MS.

9.5.4 Field Ionization Mass Spectrometry (FI-MS)

The molecules having less parent ion can be determined using this technique. FI-MS has foil type field ionization source which is attached to the mass analyzer and the data is recoded by using the detector. The modern technique of FI-MS has been introduced which is FD-MS. In FD-MS, the sample is evaporated by field ion emitter and then introduced in high electric field.

9.5.5 Fast Atom Bombardment Mass Spectrometry (FAB-MS)

In this technique there is bombardment of the compound with high energy neutral particles. For example, argon and xenon. This technique is commonly used for the estimation of vitamins, nucleotides, and peptides.

9.6 Applications

1. In the field of biotechnology, it is used for the analysis of proteins, peptides, oligonucleotides.
2. In the field of pharmaceutical industry, it is used for the drugs discovery, combinatorial chemistry, pharmacokinetics, drug metabolism, purity of compounds, drug stability, drug–polymer interaction.
3. In the field of clinical and medical sciences, it is used for neonatal screening, hemoglobin analysis, drug testing, detection of steroids, disease diagnosis, determination of anesthetics.
4. In the field of environmental sciences, it is used for the detection of various types of pollutants in atmosphere, environment, and water.
5. In the field of polymer chemistry, it is used for the characterization of polymers.
6. In the field of food chemistry, it is used for the find out the adulteration in food stuff, detection of toxicants in food.
7. In the field of medicinal chemistry, it is used for the determination of:
   - Accurate molecular weight can be measured.
   - Purity of sample, unknown sample, and other side products.
   - Reaction monitoring.
   - Protein digestion, chemical modification, enzyme activity.
   - Structural elucidations.
   - By the fragmentation of unknown products and natural products.
   - Mechanism of actions of the compounds can be identified.
   - The compounds having either non-covalent or the covalent interactions.
   - Active site identifications.
   - Quantitative analysis.
   - Impurity profiling for the bulk drugs.

9.7 Advantages

1. It is high sensitivity.
2. It requires the small size of sample.
3. It is time saving.
4. When it used in combination with other methods, it exhibits high sensitivity and acceptability.
5. It can be used for differentiation the isotopes of the atom.

9.8 Disadvantages

1. Only pure compounds can be handled readily.
2. Compounds that are non-volatile in nature cannot be handled by MS.

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Nuclear Magnetic Resonance Spectroscopy 10

Abstract

Nuclear magnetic resonance (NMR) spectroscopy is usually combined with infrared (IR) spectroscopy for the complete analysis of the structure of an unknown molecule. IR spectroscopy is used to detect a functional group in the sample, whereas NMR spectroscopy detects number of atoms and their type in sample. NMR technique can detect many nuclei but mostly identifies carbon-hydrogen frameworks. In this chapter, we have comprehensively discussed the NMR spectroscopy, its types, basic mechanism along with its instrumentation, applications, advantages, and disadvantages.

Keywords

Nuclear shielding · NMR spectra · Components of NMR spectroscopy · Types of NMR spectra

10.1 Introduction

NMR spectroscopy also known as magnetic resonance spectroscopy (MRS) is the most powerful analytical technique among all spectroscopic techniques. It visualizes single atom and molecule in various media, both in solution state and solid state. NMR is a nondestructive technique and it gives molar response that can be used for structure elucidation and quantification. Magnetic interactions which occur between the active nuclei and NMR along with covalent bonds result into spin–spin coupling. We can detect the space interactions by using the effect of nuclear Overhauser enhancement (NOE). These interactions are used for the elucidation of three-dimensional structure. However, 1-dimensional and 2-dimensional NMR data can also be collected. The 1-D NMR experiments are ^{1}H, ^{13}C, ^{19}F, and ^{31}P. The 1-D NMR techniques are used to study the chemical shift, spin–spin coupling, and intensities. Information regarding protons and its environment can be obtained with the help of chemical shift. Nuclei closer to each other exert an effect on other's

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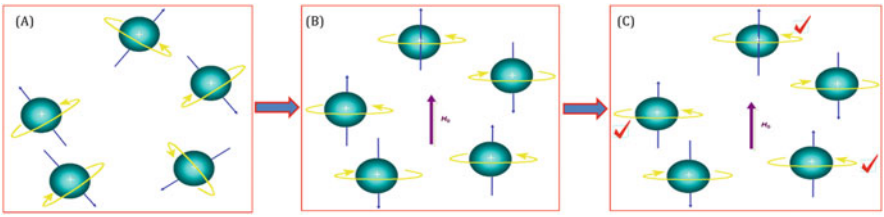

Fig. 10.1 Schematic representation of distribution of nuclear spins

effective magnetic field. This effect is shown in NMR spectrum when the nuclei are non-equivalent. If the distance between the non-equivalent nuclei is less than or equal to three bond lengths then this effect can be observed, the effect is called indirect spin–spin coupling. Radio waves are the energy source in NMR that has longer wavelengths, and hence has lesser energy and frequency. When low-energy radio waves interact with any molecule, then they change the nuclear spins of some elements, including ^{1}H and ^{13}C. The nuclear spins distribution is mostly random if there is no external magnetic field (Fig. 10.1a). But when an external magnetic field is applied then it aligns the nuclear magnetic moments with the applied field either in parallel or anti-parallel manner (Fig. 10.1b). If parallel alignment happens then nuclear magnetic moments will be slightly more (Fig. 10.1c).

Resonance: It is the process of amplification which occurs when the frequency of the applied force shows harmony with the system's natural frequency. It is related to the alteration in nuclear spin of systems from lower energy state to a higher energy state by the process of energy absorption. This can be done by the creating a magnetic field around the nuclei.

Spin: it is a number which is associated with the quantum mechanical property of nuclei. Its value must be an integer if mass number is even or half integer if mass number is odd.

Spin-lattice relaxation: Lattice is a term used for the nuclei which is held in the framework, whereas lattice field is generated when a magnetic field is created by vibration of sample nuclei. Magnetic field which shows equilibrium with the ground state energy field is known as spin-lattice relaxation.

Spin–spin relaxation: It is simply the interaction between the neighboring nuclei having same frequencies but having different magnetic quantum. In this state, the nuclei can exchange their quantum state with the nucleus which is excited in the lower energy state and with the excited nucleus which is relaxed to lower energy state.

Spin–spin coupling: It is the effect of spin state of one nucleus on the energy of another nucleus which is responsible for peak splitting. This effect is transmitted by intervening bonding electrons. Because of this effect, lines of NMR spectra are split.

Nuclear Overhauser enhancement (NOE): When spectrum of proton-decoupled ^{13}C is obtained then intensity of resonance of some carbon atoms is increased significantly than those observed in proton-coupled experiment. Carbon atoms which are attached directly to the hydrogen atoms are enhanced the most and

when more hydrogen atoms are attached (via saturation) this enhancement is increased. This effect is known as nuclear Overhauser effect and the degree of enhancement in the signal is called NOE.

Nuclear Shielding: The applied magnetic field is not equal to the magnetic field around the nucleus because the electrons present around the nucleus shield it from the applied field. The difference of both these magnetic fields is known as nuclear shielding whereas

$$\text{Chemical shift} = \text{nuclear shielding}/\text{applied magnetic field}$$

10.2 Types of Nuclear Shielding

1. *Local shielding*: it is the field created by local electrons on that nucleus.
2. *Low range shielding*: it is a field which I created by π-electrons that are not associated with the nucleus.

10.3 Intensities of Resonance Signals

These are of two types which are as follows:

10.3.1 ^{1}H NMR Signal Intensities

^{1}H-NMR is used to detect the type and number of H atoms in a molecule. The intensity or integral of a signal in the spectrum is thought to be the area under that signal. The ratios of protons present in a molecule of the compound can be determined with the comparison of signal intensities in the spectrum. If spectrum shows multiple readings then whole group of peaks should be integrated separately. The signal intensity is an important parameter to determine the structure of molecule and for quantitative analysis of molecule.

10.3.2 ^{13}C NMR Signal Intensities

^{13}C-NMR is used to detect the type of carbon atoms in the molecule (Fig. 10.2). ^{13}C-NMR signal is valuable in determining the total number of C-atoms responsible for the signal. Practically, low abundance and less sensitivity of the ^{13}C isotope will have an effect on the quantification of number of carbon atoms in the molecule. Therefore, the signals of carbon are usually not integrated in the spectrum of ^{13}C NMR. The quantification of ^{13}C signal can be made possible

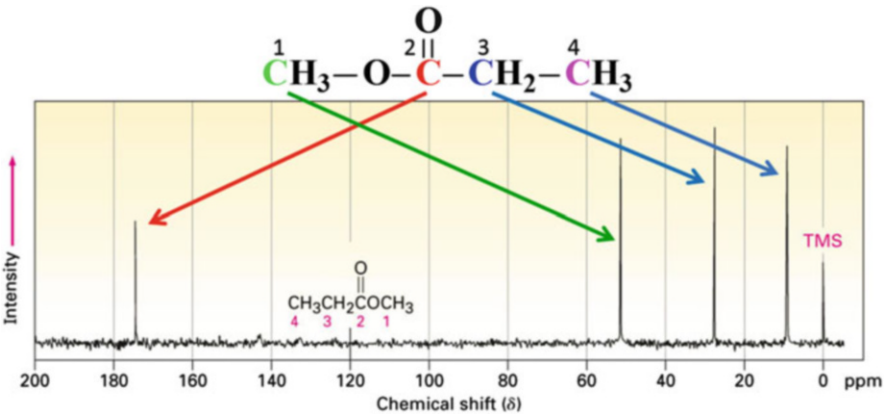

Fig. 10.2 A schematic representation of ^{13}C spectrum of methyl propionate obtained from NMR

with suppression of the NOE, high digital resolution, rate of pulse repetition which should not be too fast, high pulse power, and small spectral width.

10.4 One-Dimensional NMR Spectroscopy

The spectrum of 1D NMR spectroscopy has 2 dimensions, x-axis which is the frequency axis and y-axis which corresponds the signal intensities.

10.5 Two-Dimensional NMR Spectroscopy

In the spectrum of 2D NMR both x-axis and y-axis represent frequency and intensity is shown on the z-axis. In the spectrum of 2D J- resolved NMR, the chemical shifts are present along x-axis and coupling constants are plotted along y-axis. If both of the axes correspond to chemical shifts, then it is known as 2D (shift) correlated NMR spectrum. The correlations can be homo nuclear (^1H–^1H) or it can be hetero nuclear ($^1H/^{13}C$).

10.6 NMR Spectra

It is actually a graph between the intensity of peak and its chemical shift which is measured in ppm.

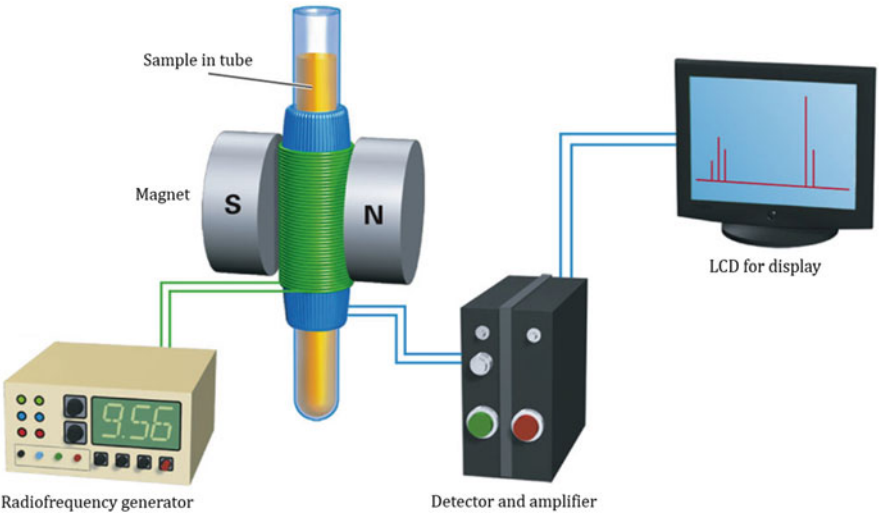

Fig. 10.3 Schematic representation of components of NMR spectrophotometer

10.7 Components of NMR Spectroscopy

General representation of basic components of NMR spectrophotometer has been illustrated in Fig. 10.3 and the detail description of individual component of NMR has been discussed in the following sub-sections.

10.7.1 The Magnet

In the absence of magnetic field, all nuclear spin states are well populated and therefore there is no net polarization. Therefore, an external magnetic field is applied to achieve a preferential population of nuclear energy spin states. A higher magnetic field leads to greater separation of energy levels and greater polarization at equilibrium. The magnet may be a powerful permanent magnet or a cryogenically cooled superconducting electromagnet. Both of these magnets align the nuclear spin in the sample.

10.7.2 A Radiofrequency Oscillator

It consists of radiofrequency synthesizers and amplifiers. They generate a pulse sequence containing radiofrequency pulses of specific frequency, phase, amplitude, shape, and time duration. Multiple radiofrequency oscillators are required as some

NMR experiments need simultaneous application of radiofrequency pulses having different frequencies.

10.7.3 The Sample Holder

It is used to load the sample under analysis. Mostly a glass tube is employed for the holding of both liquid and solid sample.

10.7.4 A Radiofrequency Receiver

It consisted of some components that are preamplifier, amplifier, mixer, and a converter which converts analog into digital.

10.7.5 A Recorder

A computer is used to display the results on its screen and record the data.

10.8 Solvents Used in NMR Spectra

The solvents should be:

- Chemically inert.
- Show magnetic isotropy.
- Must be volatile.
- And hydrogen atoms should be absent.

Most commonly used solvents are cadmium chloride, carbon tetrachloride, deuterium oxide, carbon disulfide, and hexa deuteriobenzene.

10.9 How to Interpret NMR Spectra

Before the interpretation of NMR spectra, it is very important to understand the role of chemical shift and reference peak during the interpretation of NMR spectra. Role of chemical shift and reference peak has been briefly elaborated in Fig. 10.4a. Similarly, how the chemical shift is moved across the NMR spectra, has been described in Fig. 10.4b.

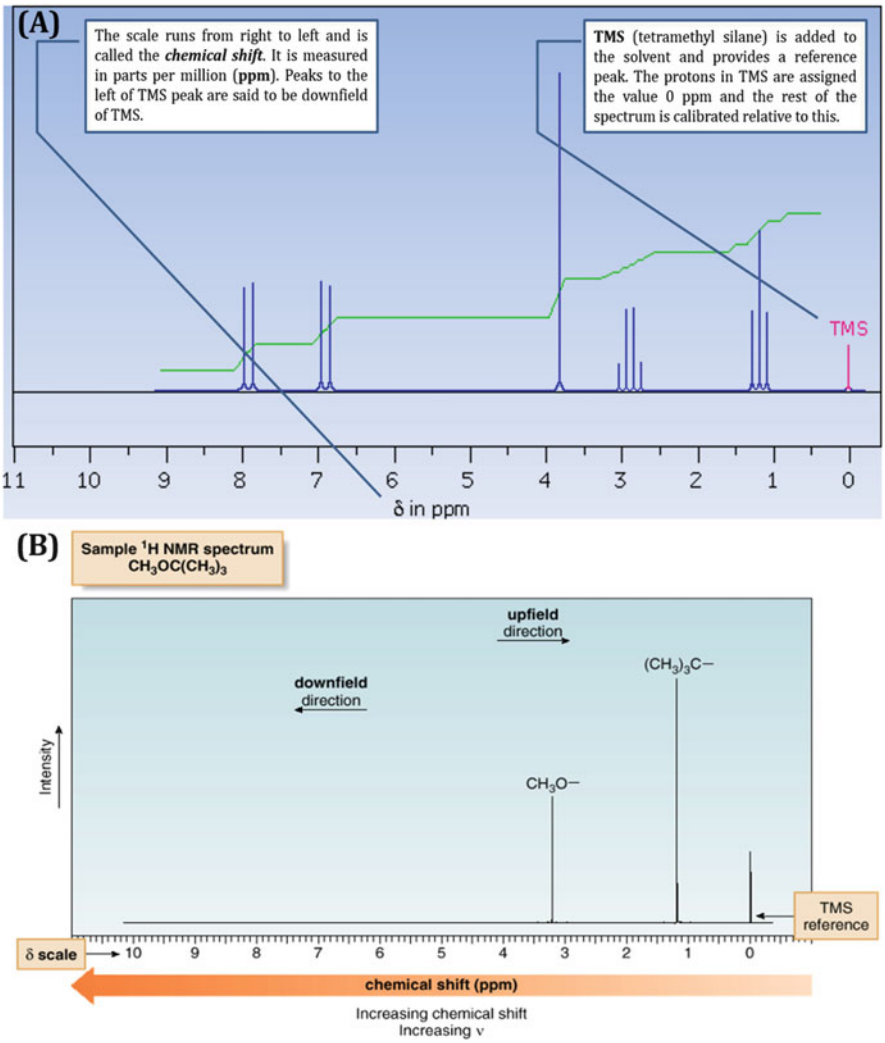

Fig. 10.4 The schematic representation of interpretation of NMR spectra

10.10 Types of NMR Spectra

There are many types of NMR spectra (Fig. 10.5) that depend on many factors including (a) type of the instrument being used, (b) nucleus and its type that is involved, (c) physical state of the analyte, (d) environment around the sample nucleus, and (e) object of data collection. Commonly, NMR spectra are of two types.

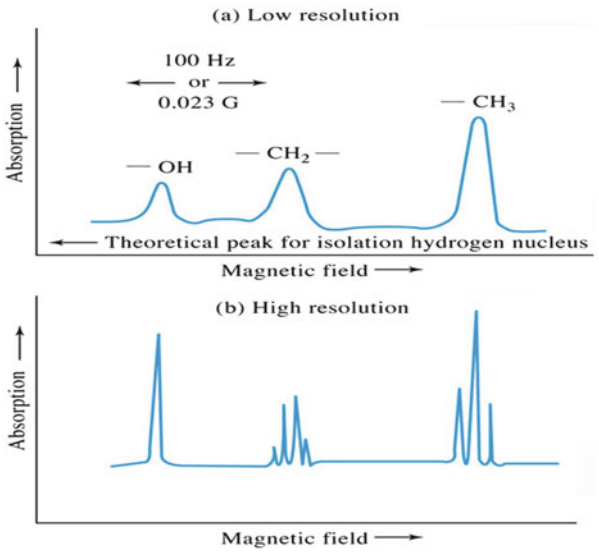

Fig. 10.5 Schematic representation of types of NMR spectra

10.10.1 Wide-Line Spectra

This is the spectra in which the bandwidths between lines are so large that we can elucidate the fine structure of the analyte because of chemical environment. Each species shows its own single peak.

10.10.2 High-Resolution Spectra

Most NMR spectrums have a high resolution and they are collected through instruments which differentiate very small frequency differences that can be of 0.01 ppm or less. For example, in the lower-resolution spectrum of ethanol, 3 peaks can be seen which formed due to the absorption by the protons of CH_3, CH_2, and OH, whereas in the higher resolution spectrum, 2 out of 3 peaks are resolved into the additional peaks.

10.11 Advantages

1. High resolution.
2. High flexibility.
3. Non-destructive method.
4. Analytically tractable.
5. Highly predictable for small molecules.

10.12 Disadvantages

1. Low accuracy.
2. Highly expensive.
3. Not able to differentiate the same compounds.
4. Time consuming.

10.13 Applications

1. In the field of natural product chemistry, NMR can be used for the structural and chemical elucidation of isolated compounds.
2. In the field of synthetic and organic chemistry, it can be used as an analytical tool of choice by synthetic and organic chemists.
3. It can be used for the determination of study of dynamic processes like reaction kinetics and study of equilibrium.
4. It is also used for 3-dimensional studies of proteins, protein-ligand complexes, polysaccharides, DNA < RNA and protein-DNA complexes.
5. In the field of drug design, it is also used for the determination of structure activity relationship.
6. In the field of medicine, it is also used for the detection of tumors, amino acids, proteins. RNAs and DNAs. It is also used in metabolic fingerprints from biological fluids.
7. It can also be used for the purity determination of any compound provided that molecular weight of structure of that compound is known.
8. It can also be used for diagnostic purposes, e.g., to determine the metabolic products in body fluids.
9. It is widely used for the study liposomes.
10. This technique allows easy and non-destructive of many components involved in biodiesel standardization, e.g., water, phosphorus, alcohol, and glycerol content.
11. In the field of food sciences, this technique is used to analyze moisture content, solid fat content, etc.

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Introduction to Chromatographic Techniques

11

Abstract

In this chapter, we have described the brief overview of the most important analytical technique i.e., chromatography which is used for the separation, identification, and purification of the components present in a complex mixture. We have briefly discussed in detail the basic principle involved in chromatography during the phenomenon of separation, identification, and purification of the components present in complex mixture. Moreover, various types of chromatography have also been discussed here.

Keywords

Principle of chromatography · Types of chromatography · Mechanism of separation

11.1 Introduction

Chromatography is one of the most important analytical techniques that is used for the separation, identification, and purification of the components present in a complex mixture that contains different sizes and/or molecular weights of individual components. Chromatography is used for both qualitative and quantitative analysis of the analyte present in complex mixture. Chromatography is also known as the science and art of separation of components and/or analyte present in complex mixture which involves the physical separation of component of interest present in mixture into its individual components. The components and/or substances that are going to be separated, identified, and/or quantified should be physically mixed together, but not chemically combined. Here are some of the most common examples of physical mixtures:

- Water suspended in air is a physical mixture of fog.
- Mixture of gases present in the universe is the physical of air.

M. S. H. Akash, K. Rehman, *Essentials of Pharmaceutical Analysis*,
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- Mixture of cereal and milk is known as bowl of cereal.
- Mixture of water, soda syrup, and CO_2 gas is known as soda pop.
- Mixture of sugar, water, and flavor crystals is known as kool-aid.

11.2 Principle of Chromatography

Chromatography is based on the principle where the molecules, components, and/or substances present in mixture are applied onto or into the solid surface and/or sometimes fluid stationary phase and then separated from each other while moving from one side of the solid surface to another side with the aid of a mobile phase. The factors that may effect on this separation phenomenon include molecular characteristics related to the adsorption of substance and/or components that are being separated, partition coefficient and affinity among the molecular weights and/or size of the components that are being separated. Because of the differences in the molecular weights and/or sizes of the individual components present in the mixture, some components of the mixture move slowly in chromatographic system and stay longer on stationary phase, while the others pass rapidly into the mobile phase and leave the chromatographic system faster.

11.3 Terms Used in Chromatography

11.3.1 Mobile Phase

A phase in which the sample is dissolved is known as mobile phase. It is always composed of gas, liquid, or supercritical fluid. It is very important that mobile phase should be compatible with all the components present in the mixture that are going to be separated.

11.3.2 Stationary Phase

The phase in which the mobile phase is forced through is known as stationary phase. This phase is always composed of a "solid" phase or "a layer of a liquid adsorbed on the surface a solid support."

11.3.3 Supporting Medium

A solid surface on which the stationary phase is bounded or coated is known as supporting medium. When the mobile phase is allowed to pass on it, the analyte will

distribute itself between the two phases based on the affinity and/or partition coefficient.

11.3.4 Eluate

It is that component which is obtained after the completion of chromatographic process.

11.3.5 Eluent

It is the carrier portion of the mobile phase. It moves the analytes through the chromatographic apparatus.

11.3.6 Elution

It is the phenomenon of extracting a substance and/or component that is adsorbed on another surface by moving it with the solvent.

11.3.7 Chromatogram

A graph showing the detectors response as a function of elution time, band's shapes, position, resolution, is known as chromatogram.

11.3.8 Retention Time

The time needed after the injection of sample into the column for an individual solute to reach to the detector is known as retention time.

11.4 Types of Chromatography

11.4.1 Based on the Nature of Mobile Phase

11.4.1.1 Liquid Chromatography

This technique is used to separate the components and/or substances present in liquid samples with a liquid solvent that is known as mobile phase and a column composed of solid beads that is also known as stationary phase.

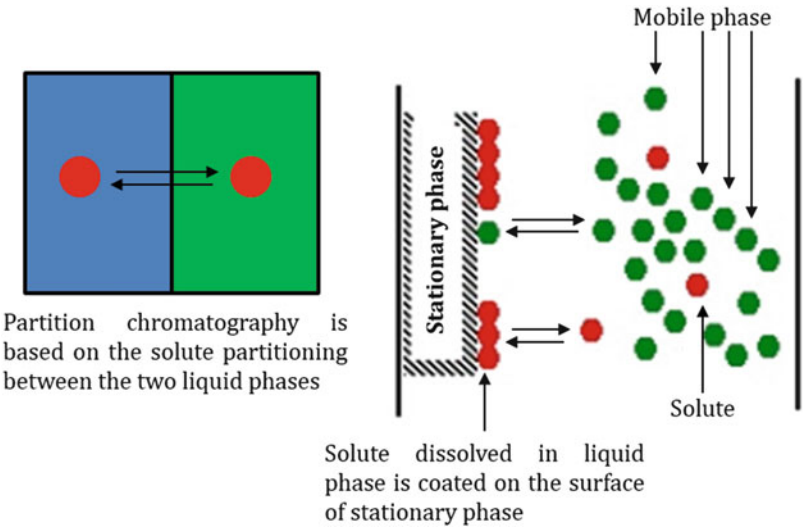

Fig. 11.1 Schematic representation of partition chromatography

11.4.1.2 Gas Chromatography

This chromatographic technique is used to separate vaporized samples with the help of a carrier gas which acts as mobile phase and a column that is composed of a liquid or of solid beads (stationary phase).

11.4.1.3 Paper Chromatography

This chromatographic technique is used to separate the dried liquid samples with the help of liquid solvent that acts as mobile phase and a paper strip that acts as stationary phase.

11.4.1.4 Thin Layer Chromatography

This chromatographic technique is used to separate the dried liquid samples with the help of liquid solvent that acts as mobile phase and a glass plate that is covered with a thin layer of alumina or silica gel that acts as a stationary phase.

11.4.2 Based on the Mechanism of Separation

11.4.2.1 Partition Chromatography

In this type of chromatographic technique, the components and/or substances of interest are distributed more likely into two mobile phases because of the differences in partition coefficients. Molecules will partition into the stationary phase based on their affinity towards the stationary phase and eventually partition into the mobile phase again. A schematic representation of partition chromatography has been described in Fig. 11.1.

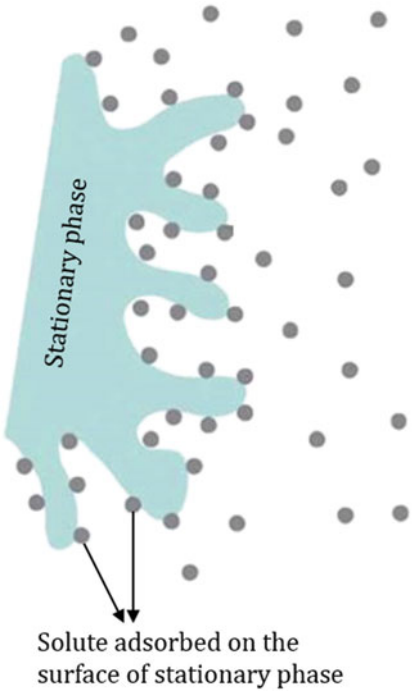

Fig. 11.2 Schematic representation of adsorption chromatography

11.4.2.2 Adsorption Chromatography

Adsorption chromatography is based on the principle of adsorption phenomenon. In adsorption chromatography, the separation of the components present in mixture is based on the interaction of the adsorbate with the adsorbent. The adsorbent is the solid surface of stationary phase and the adsorbates are the molecules of interest present in the sample mixture and are getting adsorbed on the adsorbent (solid stationary phase). The phenomenon of adsorption chromatography is very similar to that of the partition chromatography. Adsorption of components and/or substances of interest present in mixture, just take place on the solid surface of the stationary phase. A schematic representation of adsorption chromatography has been described in Fig. 11.2.

11.4.2.3 Ion-Exchange Chromatography

It is the type of chromatographic technique, the polar molecules and/or ions are separated based on their affinity to the ion exchanger (that acts as a stationary phase). Ion-exchange chromatography is used for the separation of almost any kind of charged molecule including large amino acids, small nucleotides, and proteins. Ion-exchange chromatography is used for the separation of either cations or anions. The separation phenomenon involved in ion-exchange chromatographic technique is based on the relative strength of ionic bond. A schematic representation of ion-exchange chromatography has been described in Fig. 11.3.

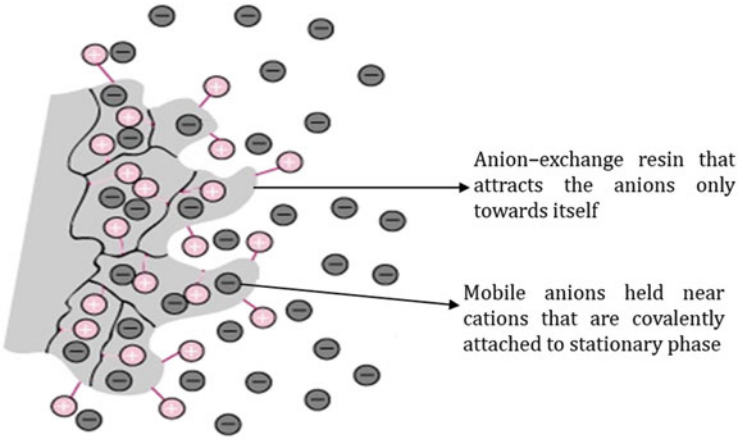

Fig. 11.3 Schematic representation of ion-exchange chromatography

11.4.2.4 Molecular Exclusion Chromatography

This chromatographic technique is also known as gel permeation chromatography size exclusion chromatography and/or gel filtration chromatography. The phenomenon of separation of the components of interest present in the sample mixture is based on the size and/or molecular weight of individual component of interest present in the mixture by the mechanism of filtration through the gel which acts as stationary phase consisting of heterosporous (pores of different sizes) cross-linked polymeric gels or beads. Small molecules get trapped into the pores of stationary phase more rapidly as compared to that of the large molecules that do not entered into the gel easily and cover the longer distance in stationary phase. The flow of the component of interest through the gel (stationary phase) is retarded according to their size. The large molecules are eluted out (cannot enter into the gel or stationary phase due to their large size or molecular weight). It is applied to separate the large molecular weight macromolecular complexes such as proteins and industrial polymers for semi-preparative purifications and various analytical assays. This technique is used for the purification and studies of desalting, protein-ligand, protein-folding, and copolymerization phenomenon. A schematic representation of molecular exclusion chromatography has been described in Fig. 11.4.

11.4.2.5 Gel Electrophoresis

Gel electrophoresis is the type of chromatographic technique which is used to separate protein molecules, DNA, and RNA according to their molecular sizes. In this technique, the molecules that are going to be separated are pushed by the involvement of an electrical field through a gel that contains small pores and acts as stationary phase. The samples containing the components of interest are loaded into the wells at the one end of a gel and an electric current is applied to pull them through the gel. The fragments of the components of interest are negatively charged, so they move towards the positive electrode when an electric current is applied. Because all fragments of component of interest have same amount of charge per

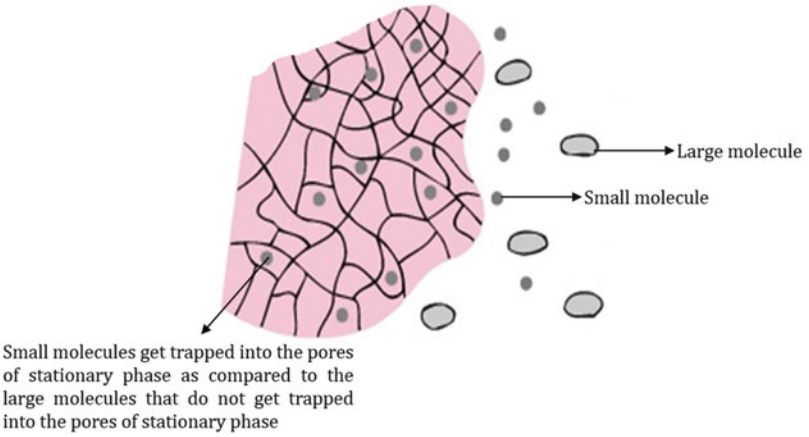

Fig. 11.4 Schematic representation of molecular exclusion chromatography

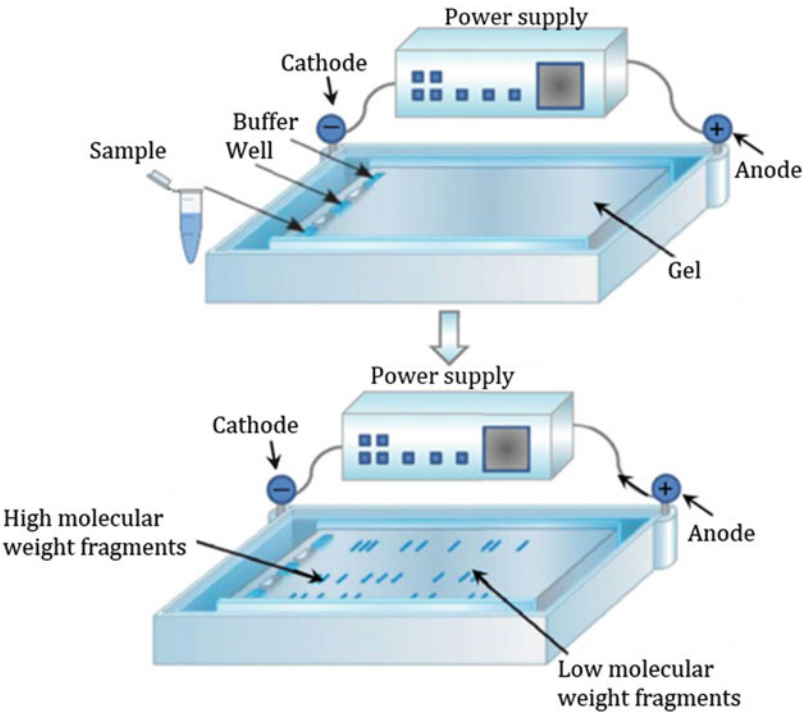

Fig. 11.5 Schematic representation of gel electrophoresis apparatus

mass ratio, therefore, the small fragments move through the gel faster and more efficiently as compared to that of the large ones as shown in Fig. 11.5.

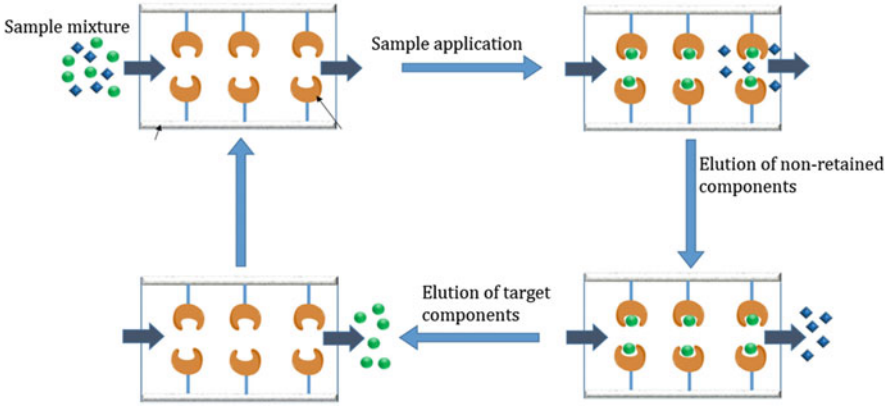

Fig. 11.6 Schematic representation of affinity chromatography

11.4.2.6 Affinity Chromatography

It is the type of chromatographic technique which is based on the specific binding interaction in adsorption that depends on the specific affinity between an immobilized ligand that is fixed in the separation material and its binding partner (desired component present in the mixture). The interaction between the mobile phase and stationary phase is typically reversible and purification and/or separation is achieved through a biphasic interaction with the ligand that is immobilized on a stationary surface while the target that is in mobile phase as part of a complex mixture. The most common examples that may be separated and/or purified by this technique are antibody–antigen, enzyme–substrate, and enzyme–inhibitor interactions. Affinity chromatography is used for selective purification of a molecule from complex mixtures that is based on highly specific biological interaction between the two molecules. A schematic representation of affinity chromatography technique has been described in Fig. 11.6.

11.5 Applications

1. In pharmaceutical industry, chromatographic techniques are used to analyze the samples for the presence of trace elements, separation of compounds based on their molecular weight and element composition, detection and identification of unknown compounds and purity of mixture.
2. In hospitals, chromatography is one of the best tools for the detection of narcotics and alcohols in blood and/or urine sample.
3. Environmental protection agencies use chromatographic tools for the detection of pollutants present in the environment.
4. In food industry, chromatographic techniques are used for the detection of spoilage, presence of additives in food and quality of nutritional components present in food.

5. In forensics, chromatographic techniques are used for crime scene testing, forensic pathology, and arson investigation.
6. In molecular biology, chromatographic techniques are used to study the redox reactions that involve various bioorganic molecules, characterization of reaction mixtures during the study of biomolecules.
7. In metabolomics, chromatographic techniques are used in mimicking the biotransformation reactions, such as phase I oxidative reactions in drug metabolism studies and in the study of active pharmaceutical ingredients.
8. In proteomics, chromatographic techniques are used to analyze the oxidation of biomolecules such as proteins and peptides. It is also used for the purification of monoclonal antibodies, hormones, vaccines, and plasma proteins as part of their development.
9. Chromatographic techniques are also used in nucleic acids research for the identification of oxidation products of nucleosides, nucleotides, and nucleobases.

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Thin Layer Chromatography

12

Abstract

Thin layer chromatography (TLC) is a type of chromatographic technique which is used for the separation of the components present in mixture using thin stationary phase. Separation of the components depends on the competition between the adsorption of solute on stationary phase and its desorption by the solvent needed to wash out from the stationary phase. In this chapter, we have discussed the basic principle of TLC, components of TLC, its working and applications.

Keywords

Nature of phases · Principle of TLC · Types of TLC · Applications of TLC

12.1 Introduction

Thin layer chromatography (TLC) is a type of chromatographic technique which is used for the separation of the components present in mixture using thin stationary phase (supported by an inert backing). It is a solid–liquid technique in which there are two phases: one is stationary phase and the other is mobile phase. Separation of the components depends on the competition between the adsorption of solute onto the solid surface (stationary phase) and its desorption by the solvent needed to wash out from the stationary phase. In TLC, stationary phase is always in solid state while the mobile phase is always in liquid state.

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12.2 Principle

In 1949, the two scientists, namely Mein Hard and Hall proposed TLC technique using starch as a binder to separate the inorganic ions present in the mixture. While, Izmailov and Shraiber proposed TLC principle and Stahl designed the TLC equipment. TLC is based on the following principle:

1. Separation of individual component present in sample mixture is dependent on the relative affinity of individual component with stationary phase and the mobile phase.
2. If the affinity of individual component present in a sample mixture is high with mobile phase, then it travels over the surface of the stationary phase eluted out. Whereas, if the affinity of individual component present in sample mixture is higher with stationary phase, then it travels slowly and retained in the stationary phase. Thus, the separation of components in the mixture is achieved.
3. Once the separation occurs, individual components of interest are visualized as spots appeared at different points on TLC plate.

12.3 Types of Thin Layer Chromatography

12.3.1 Based on Nature of Phases

Based on the nature of phases used in TLC, it can be classified into the following two types which have been briefly described in Table 12.1.

12.3.2 Based on Purpose of Use

TLC can also be classified into the following two types based on the purpose of the application:

12.3.2.1 Analytical Chromatography

When the purification of small amount of the compound present in the mixture is required, then this type of TLC is known as analytical TLC.

Table 12.1 Difference between normal and reverse phase TLC technique

Sr. #	Parameters	Normal phase TLC	Reverse phase TLC
1	Stationary phase	Polar	Non-polar
2	Mobile phase	Non-polar	Polar
3	Component that eluted at first	Non-polar	Polar
4	Component that eluted at last	Polar	Non-polar

12.3.2.2 Preparative Chromatography

When the purpose is to monitor any progress in chemical reaction occurring in the mixture, then this type of TLC is known as preparative TLC.

12.4 Components of Thin Layer Chromatography

Following are the components of TLC:

12.4.1 Developing Chamber

It is used to create and maintain the environment for TLC.

12.4.2 Solid Support

It is used to support TLC film coated on stationary phase.

12.4.3 Stationary Phase

It is used to provide facility for the adsorption of analyte on TLC film.

12.4.4 Mobile Phase

It is a solvent system that carries the analyte present in mixture.

12.4.5 Pipette

It is used to load the samples on the stationary phase of TLC film.

12.4.6 Forceps

They are used to handle the TLC plates during and after experiment.

12.5 Working on Thin Layer Chromatography

The following steps are involved while working on TLC:

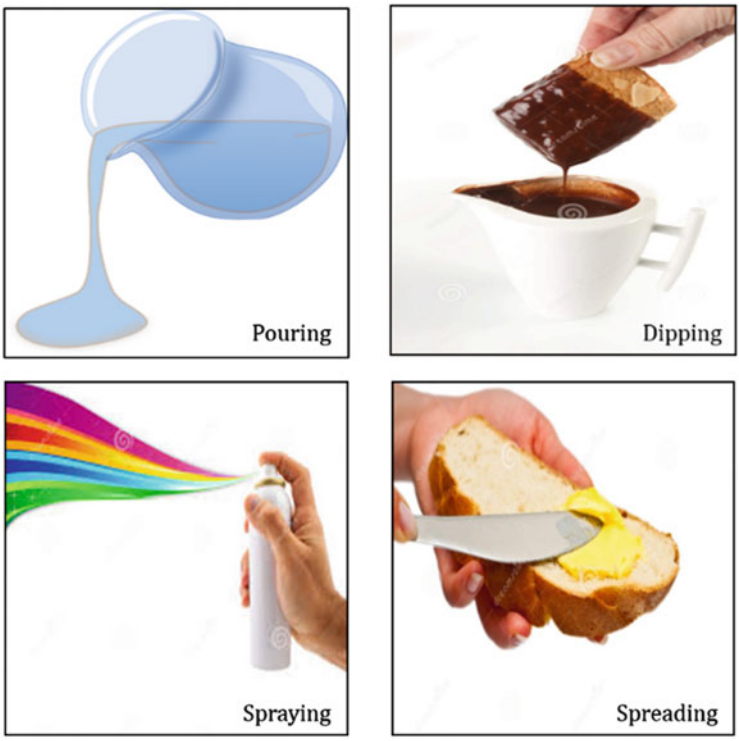

Fig. 12.1 Schematic representation of preparation of TLC plate

12.5.1 Preparation of TLC Plates

At first, the suspension or slurry of coating material is prepared and then applied on the solid surface that acts as stationary phase by one of the following methods. A schematic representation of preparation of TLC plates has been briefly described in Fig. 12.1.

12.5.1.1 Pouring

In this technique, TLC plate is kept on a level surface and the measured amount of slurry of adsorbent is put on plate. Then the plate is tilted back and forth to spread slurry of adsorbent (Fig. 12.1).

12.5.1.2 Dipping

This method was developed in 1962 by Peifer. In this technique, TLC plate is dipped in slurry of adsorbent (Fig. 12.1).

12.5.1.3 Spraying

This method was proposed by Resitsema, but nowadays this technique is not used. In this technique, pointer sprayer is used to distribute slurry of adsorbent on TLC plate (Fig. 12.1).

12.5.1.4 Spreading

In this technique, the slurry of adsorbent is placed on applicator and the applicator is either moved on the stationary phase or it is kept static and TLC plate is pushed and/or pulled through the applicator (Fig. 12.1).

12.5.1.5 Activation of Adsorbent

Once the TLC plate is prepared, it is dried by keeping it in air for 30 min and then, it is kept in oven for another 30 min at 110 °C for the complete evaporation of solvent. This drying technique activates the adsorbent layer on TLC plate. The most commonly used active layers on TLC plates are silica gel and alumina. These adsorbents on TLC plate can be activated by heating at 150 °C for 4 h.

12.5.1.6 Purification

Sometimes, the adsorbent material may contain any impurity, e.g., silica gel may contain iron as an impurity. It is very important that the adsorbent material must be purified.

12.5.1.7 Preparation of Mobile Phase

The mixture of two or more solvents of different polarity with different proportions is often used for the separation of components present in mixture as compared to that of chemically homogenous solvents.

12.5.1.8 Marking on TLC Plate

A line about 1 cm is marked below the top of TLC plate and then a small point is marked on either side of spotting point about 2 cm from the bottom. Care must be taken during the marking on TLC plate so that the adsorbent material should not be removed from the sides, top, or bottom of TLC plate. A schematic representation of marking on TLC plate has been described in Fig. 12.2.

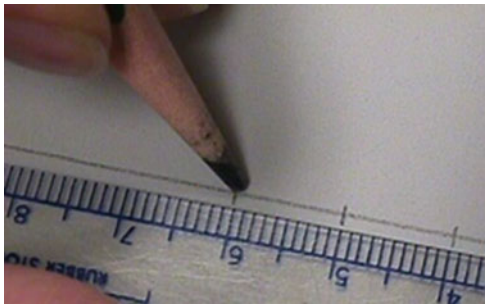

Fig. 12.2 Schematic representation of marking on TLC plate

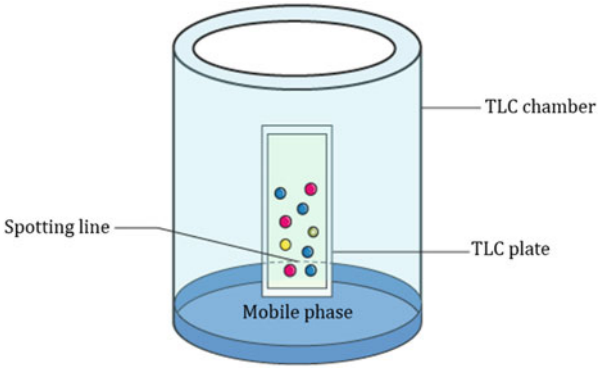

Fig. 12.3 Schematic representation of TLC chamber

12.5.1.9 Preparation of Sample and Standard Solutions

Before the preparation of sample and standard solutions for TLC experiments, stock and dilutions are prepared and calibrated. One dosage unit or composite of dosage units of analyte is placed in small container, ground to make its power and transfer to a suitable vessel. Then, an appropriate solvent is used to dissolve the active ingredient (analyte).

12.5.1.10 Spotting on TLC Plate

For spotting the sample and standard solution on TLC plate, capillary tube or micropipette or calibrated glass tube or alga micro syringe is used. It is very important that the solvent used for the preparation of sample must be non-polar and volatile in nature so that after spotting the sample, the solvent may evaporate from the spots before developing.

12.5.1.11 Development

Mobile phase is added in TLC chamber (Fig. 12.3) in such a way that the bottom is covered to a height of at least 1 mm by the mobile phase.

12.5.1.12 Drying

Once the development of TLC chamber is done, the solvent (mobile phase) is allowed to reach a proper distance which is also known as solvent front. This phase may take 20–40 min. After the development of chromatogram, TLC plate is taken out and solvent front is marked on TLC plate. Then TLC plate is allowed to be air dried.

12.5.1.13 Detection

It can be done either by specific or non-specific methods. If the analyte is colored, then it can be visually detected easily (Fig. 12.4) but for the detection of colorless analyte on TLC plate, two types of techniques are used:

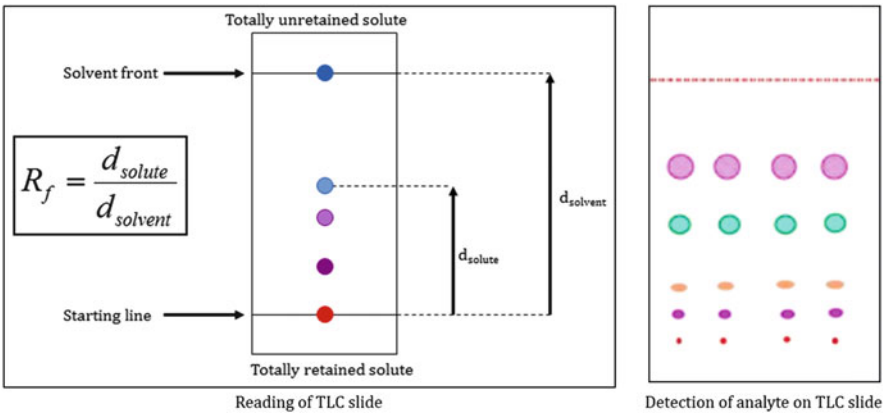

Fig. 12.4 Schematic representation of reading of TLC slide and detection of analyte on TLC slide

Table 12.2 Color formation of amino acids with ninhydrin and stannus chloride on TLC slide

Sr. #	Type of amino acid on TLC slide	Color observation of TLC slide
1	Glycine	Reddish brown
2	Leucine	Reddish orange
3	Glutamine	Pink
4	Proline	Yellow
5	Tryptophan	Pinkish violet

Destructive Technique

In this technique, specific reagent, e.g., ninhydrin and stannus chloride is used in the form of spray on TLC slide. Ninhydrin and stannus chloride react with analyte and produce specific color depending on the nature of analyte (Table 12.2).

Non-destructive Technique

In this technique, different methods are used. For example, for the detection of radioactive materials, Geiger Muller counter is used, whereas for the detection of fluorescent compounds, UV chamber is used. Iodine chamber is also used for the detection of analyte on TLC slide.

12.6 Advantages

1. TLC is a cheaper chromatographic technique.
2. The purity standards of the given sample can be easily assessed.
3. The separation process is faster and the selectivity for components is higher.
4. TLC helps to isolate the most of the components.

5. TLC helps with the visualization of separated components spots easily.
6. TLC is a simple process because of its short development time.
7. TLC helps to identify the individual components easily.

12.7 Disadvantages

1. It cannot differentiate between the enantiomers and some isomers.
2. Stationary phase in TLC is no longer stable.
3. Results produced by TLC are very difficult to be reproduced.
4. Only soluble components of interest present in mixture can be separated.
5. By TLC, only qualitative analysis is possible.
6. As TLC operates in open system, environmental factors like temperature and humidity may interfere with the overall results.

12.8 Applications

1. It is used for the separation of inorganic ions from the sample solution.
2. It is widely used for the determination of molecular weight of unknown compounds.
3. It is widely used in pharmaceutical and natural product analysis.
4. It is used for the separation of active pharmaceutical ingredients (API) from the mixture and sample solutions.
5. It is also used for the identification of API from the mixture during the synthesis of API.
6. It is also used in the investigation of pharmacokinetic studies of pharmaceutical products.
7. It is also used for the determination of essential oils in herbal drugs and assay of herbal medicines.
8. It is widely used in environmental analysis for the detection of various pollutants present in environment.
9. It is widely used in qualitative and quantitative analysis purpose.
10. It is also for the isolation and identification of carotenoid pigments.
11. It is also used for the detection of proteins and peptides.
12. It is also used for the identification of pesticides and toxins present in food items.
13. It is also used for the separation of carbohydrates, vitamins, lipids, etc. present in food items.
14. It is used for the screening and detection of drug abuse in urine samples.
15. In clinical laboratories, TLC is widely used for separation of carbohydrates in biological samples.
16. TLC is the most widely used for the routine analysis of porphyrins.
17. TLC is also used for the detection of homocysteine, neopterin, lipids, and protein antigens.

18. TLC is also used for the detection and separation of heavy metals and their complexes.

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Column Chromatography

13

Abstract

Column chromatography is a common chromatographic technique. It is a type of adsorption chromatography that is widely used for the separation of individual components of interest present in mixture. This technique can be used on small as well as on large scale for the isolation and purification of components of interest. This chapter briefly describes the basic principle involves in it, its, working, factors that may affect on its working. Moreover, advantages, disadvantages, and applications of column chromatography have also been discussed in this chapter.

Keywords

Principle of column chromatography · Working of column chromatography · Applications of column chromatography

13.1 Introduction

An American chemist D.T Day was the first scientist who introduced this technique in chemical analysis in 1900 while, in 1906, Polish botanist M.S. Tswett investigated some plant pigments using adsorption columns. A schematic representation of this technique has been illustrated in Fig. 13.1. This chromatographic technique is widely used for both the separation and purification of solid and liquid components of interest present in mixture. Column chromatography is actually a solid–liquid technique, where solid is the stationary phase and liquid is the mobile phase.

13.2 Principle

A mixture or compound that needs to be separated is dissolved first in mobile phase which is then introduced from the top of the column. Components present in the mixture then move at different rates depending upon their relative affinities towards

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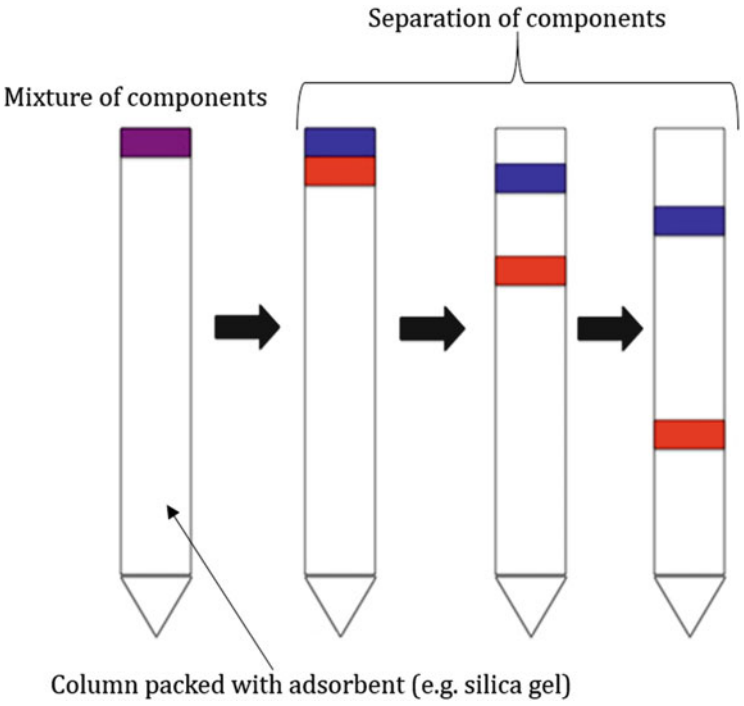

Fig. 13.1 Schematic representation of column chromatography

the stationary phase. The components having lower adsorption rate and less affinity with stationary phase will move faster as compared to those components having more adsorption and more affinity with stationary phase. The components moving faster are eluted out first, whereas those moving slowly are removed last. From the distance travelled by solute, a retardation factor is calculated:

$$R_f = \text{Distance travelled by solute}/\text{total distance travelled by the solvent.}$$

13.3 Types of Column Chromatography

Following are the most common types of column chromatography. These techniques have been briefly described in Chap. 11.

1. Absorption chromatography
2. Partition chromatography
3. Gel chromatography
4. Ion exchange chromatography

13.4 Components of Column Chromatography

Following are the main components of column chromatography:

13.4.1 Stationary Phase

Stationary phase is solid in column chromatography which should have good adsorption property. Stationary phase should be selected properly to achieve the success of column chromatography. It depends on the following conditions:

1. Removal of impurities present in the compound.
2. Number of components to be separated.
3. Affinity differences between the components.
4. Length of the column used.
5. Quantity of adsorbent used.

A stationary phase should have the following properties:

1. Should have uniform shape and size of particles
2. Mechanically stable
3. Chemically inert
4. Allow free flow of the solvent
5. Should be colorless
6. Inexpensive
7. Free availability

13.4.1.1 Adsorbents

Silica, calcium phosphate, calcium carbonate, starch, and magnesia are the most commonly used adsorbents in column chromatography. For less polar compounds' alumina is preferred. Silica gel also gives good results for compounds having polar functional groups. Adsorbents should have the following properties:

1. Particles should be uniform in size and have spherical shapes.
2. High mechanical stability.
3. Chemically inert.
4. Useful for the separation of many compounds.
5. Inexpensive and freely available.

13.4.1.2 Mobile Phase

Mobile phase is liquid in case of column chromatography which dissolves the mixture and transfer it to column. It acts as a,

1. Solvent: to introduce the sample mixture into the column.
2. Developing agent: to separate the components of interest present in mixture in the form of bands.
3. Eluting agent to remove the separated components out of the column.

The solvent is chosen on bases of the solubility properties of the mixture. Low boiling point and polarity of the solvents are the important factors in the selection of a solvent in column chromatography. The mostly used solvents are carbon tetrachloride, petroleum ether, ether, esters, cyclohexane, acetone, toluene, benzene, and water.

13.4.2 Column

It is used to hold the adsorbent or stationary phase. It is made up of neutral glass which should be of good quality so that it cannot affect the solvent. Usually, a burette is used as a column having length and diameter ratio of 10:1, 30:1, or 100:1. Selection of the column's dimensions depends upon the number of components in the sample, type of stationary phase, sample quantity under analysis, and components affinity towards the stationary phase. A narrow column is preferred over the short and thick column to achieve better separation.

13.4.2.1 Preparation of Column

Column is first washed properly with purified water and then with acetone. Then, it is dried properly to remove the impurities. Then column is hanged along a stand with the help of clamp in such a way that its outlet facing should be downward. Column should be packed from the bottom with a cotton wool, glass wool, filter paper, or asbestos pad so that adsorbent will not fall out. After packing, a paper disc is placed on the top of the column so that the adsorbent layer is not disturbed when the sample or mobile phase is introduced.

13.4.2.2 Packing of Column

Two techniques are used for the packing of column. One is dry packing or dry filling and second one is wet packing or wet filling.

Dry Packing of Column

Column is firstly filled with the adsorbent in dry form in this method and then the solvent is flushed through the column until equilibrium is reached. Air bubbles may be entrapped between the mobile phase and stationary phase. Cracks or void space may appear in the adsorbent layer. To address these problems tapping is done while packing of the column. A schematic representation of dry packing of column has been represented in Fig. 13.2.

Fig. 13.2 Schematic representation of dry packing of column

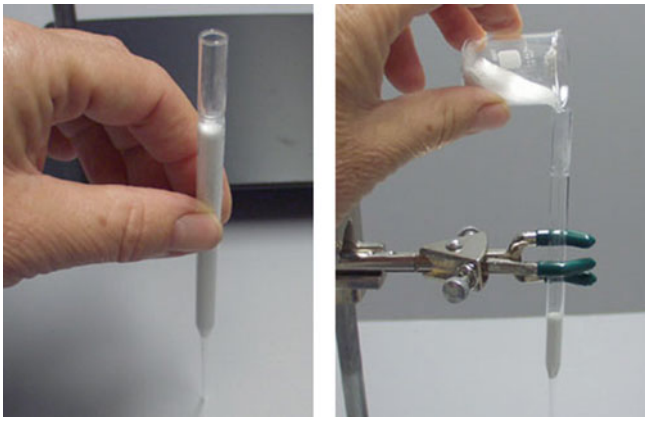

Fig. 13.3 Schematic representation of wet packing of column

Wet Packing of Column

Formations of air bubbles and cracks are the drawbacks in dry packing. Therefore, wet packing is preferred in which a slurry made up of adsorbent and solvent is generally added to the column in portions. Stationary phase (adsorbent) settles uniformly and no cracks are formed in the column. The solid settle down while the solvent remains upward. The solvent is then removed and again a cotton plug is placed in the bottom of column. A schematic representation of dry packing of column has been represented in Fig. 13.3.

13.5 Working of Column Chromatography

Column is firstly packed either by dry packing or wet packing. Then the sample is dissolved in minimum quantity of mobile phase and is introduced into the column at once. Then mobile phase is flushed through the column until 1/3 length of column is filled with solvent. By the process of elution, the components of interest are separated out from the column. A schematic representation of working of column

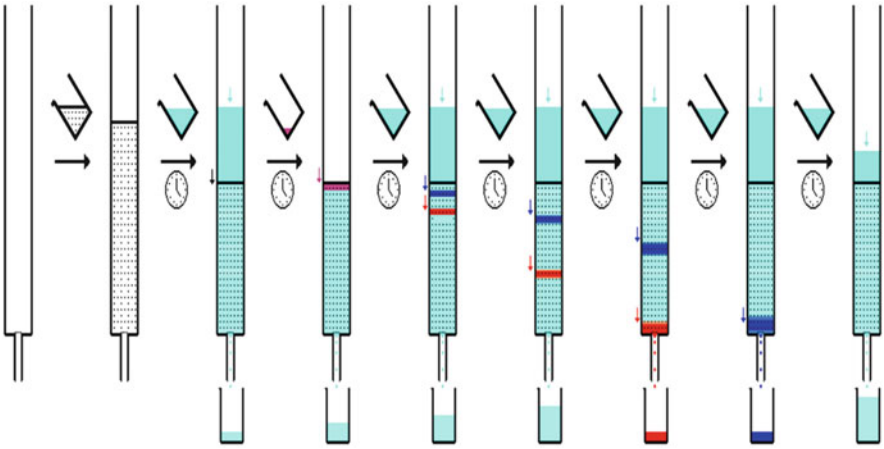

Fig. 13.4 Schematic representation of working of column chromatography

chromatography has been described in Fig. 13.4. There are two techniques which are involved in the separation process.

13.5.1 Isocratic Elution Technique

Solvents having the same composition and same polarity are used in this technique throughout the process of separation. One of the most commonly used solvents for isocratic elution technique is chloroform.

13.5.2 Gradient Elution Technique

Solvents having gradually high polarity or high strength of elution are used in this technique during the process of separation. For example, initially benzene, then chloroform, then ethyl acetate then chloroform.

13.6 Detection of Components

Detection of components of interest can be done visually if colored bands are appeared as separated components. But if the components of interest to be separated appear as colorless bands then small fractions of the eluent are collected in labeled tubes and then TLC is performed on each section separately to detect the composition of each fraction.

13.7 Factors Affecting on Column Chromatography

1. Column's dimension, length of the column should be more than its width. Normally 10:1, 30:1, or 100:1 ratios of length and diameter are used.
2. Particle size of the adsorbent should be small.
3. Proper activation of the adsorbent is needed.
4. Column's temperature should be managed properly as high temperature can enhance the process of elusion.
5. Column should be properly packed with the adsorbent and bottom should be also filled with cotton wool or anything else used for this purpose.
6. Less viscous solvents give better results.

13.8 Advantages

1. It is simple and easy technique.
2. Mixture of any type or quantity can be easily separated using this technique.
3. Many types of solvent/mobile phase can be used in this technique.
4. Automation is possible when using this technique.
5. It is an inexpensive technique.
6. This technique can be used both on small and on large scale.

13.9 Disadvantages

1. It is a time consuming process, especially when components show colorless bands.
2. In this technique, a large amount of the solvent is required for the proper elusion.
3. It is a simple technique but if automated then it becomes more complex and hence more expensive.

13.10 Applications

1. It is used to separate a mixture of compounds into its components of interest.
2. It is used for purification process.
3. The active constituents of many drugs can be isolated by using this technique.
4. It is used to determine the drug estimation in drug formulations.
5. It is also used to isolate many metabolites from the biological fluids like blood or serum.
6. Primary and secondary glycosides in digitalis leaf can be isolated by using this technique.

7. This technique is also used for the separation of diastereomers.
8. This technique is best employed for the separation of active principles of plant materials like alkaloids, glycosides, resins, tannins, and flavonoids.
9. Multistage column chromatography can be used to study the nucleotide sequences in RNA.

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High Performance Liquid Chromatography 14

Abstract

High performance liquid chromatography (HPLC) is a form of liquid chromatography which is used to separate the individual components of interest present in mixture and/or dissolved in sample solution. It is based on pumping of mobile phase through the packed column under high pressure. The basic principle involved in HPLC is based on the phenomenon of column chromatography in which the mobile phase is pumped through a packed column by applying high pressure. In this chapter, the different types of HPLC techniques on the basis of mode of chromatography, principle of separation, scale of operation, and the type of analysis have been discussed. The comprehensive instrumentation has also been discussed. At the end of the chapter, advantages and disadvantages along with its applications have been described.

Keywords

Types of HPLC · Components of HPLC · HPLC column · Sample injector

14.1 Introduction

High performance liquid chromatography (HPLC) was first proposed by Kirkland and Huber. HPLC method was developed in 1970s based on the principle of column chromatography. HPLC is a form of liquid chromatography which is used to separate the individual components of interest present in mixture and/or dissolved in sample solution. It is based on the pumping of mobile phase through packed column under high pressure (approximately 3000 psi). It is also called as "High Pressure Liquid Chromatography." The components of the mixture are separated when the sample is injected into the column. The components of the sample mixture are passed through the column at different rate depending upon the behavior of individual component of interest present in mixture and/or sample solution.

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14.2 Principle

The basic principle involved in HPLC is column chromatography in which the mobile phase is pumped through a packed column by applying high pressure. The stationary phase is present at the bottom end of the column, while the other end of the column is attached with the source of pressurized mobile phase.

14.3 Types of HPLC Techniques

HPLC techniques can be classified into the following types:

14.3.1 Based on Mode of Chromatography

1. *Normal phase HPLC mode*: In this type of HPLC technique, stationary phase is polar (e.g., silica gel), whereas the mobile phase is non-polar. In this technique, non-polar components of interest present in the mixture travel fast and eluted first as compared to that of polar components of the mixture. This is because of the less affinity of the non-polar components of interest with stationary phase. Whereas, the polar components of interest present in mixture are retained longer period of time in the column because of high affinity with stationary phase and eluted late as compared to those of non-polar components of the mixture.
2. *Reverse phase HPLC mode*: In this type of HPLC technique, the stationary phase which is used in this type of HPLC is mostly non-polar, whereas the mobile phase is usually polar. In this mode, the polar components of the mixture are eluted first, whereas the non-polar compounds retained in the column take more time to be eluted out from the column. As most of the pharmaceutical drugs are polar in nature, they do not retain in the column for longer period of time and eluted out rapidly.

14.3.2 Based on Principle of Separation

Following are the most important types of HPLC techniques that are based on the phenomenon of principle of separation. The principles of these techniques have been described in Chap. 11.

1. Adsorption chromatography.
2. Ion exchange chromatography.
3. Partition chromatography.
4. Size exclusion chromatography.
5. Affinity chromatography.

14.3.3 Based on Scale of Operation

The following two types are used on the basis of scale of operation:

1. *Analytical HPLC*: In analytical HPLC, analysis of the given sample is performed but sample recovery is impossible.
2. *Preparative HPLC*: In this analysis, the fraction collector is used in order to collect the components of the sample mixture. In this analysis the collectors are reused.

14.3.4 Based on the Types of Analysis

The following two techniques are used based on the purpose of analysis:

1. *Qualitative HPLC*: The qualitative analysis is performed to determine the characteristics of the components of a sample mixture.
2. *Quantitative HPLC*: The quantitative analysis is performed to determine the amount of the components of the sample mixture. This analysis is performed when peaks have been integrated and identified.

14.4 HPLC System

A schematic representation of HPLC system has been described in Fig. 14.1.

14.5 Components of HPLC System

Following are the major components of HPLC system:

14.5.1 Solvent Reservoir

The mobile phase that is used in HPLC mostly consists of a mixture of polar and non-polar liquid components. These liquid components have different concentrations depending on the composition of the sample used for analysis. Therefore, the components of mobile phase are separately present in a glass reservoir. This is mainly used to reserve the mobile phase. This is made up of any inert material such as glass and it does not allow the entry of the microbes. It is placed in the tray present in HPLC because if this is kept directly on the system then may be due to any spillage of solvent damage the electronic system.

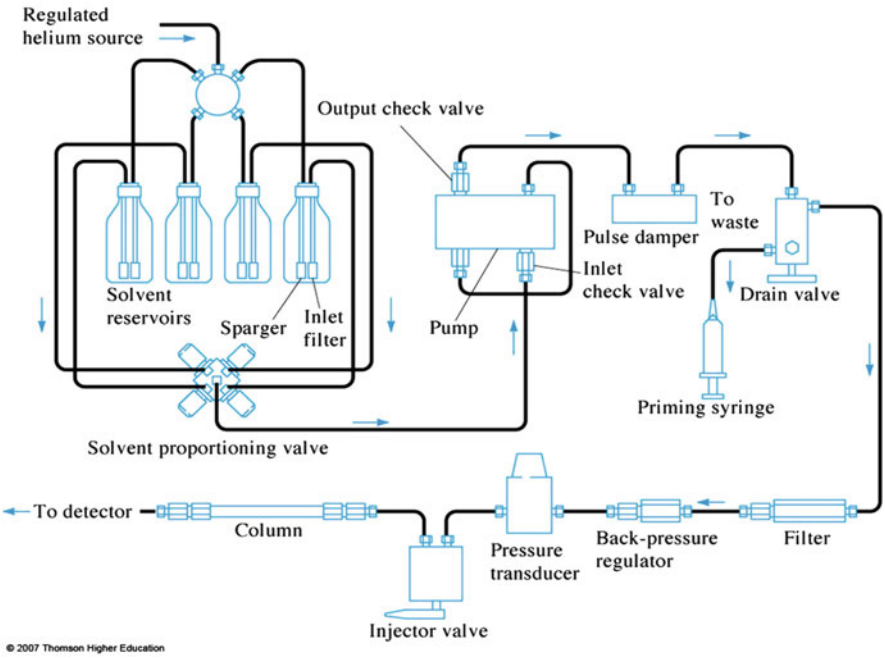

Fig. 14.1 Schematic representation of HPLC system

14.5.2 Pump

Pump is used for aspiration of the mobile phase from the solvent reservoir. Pumps force it through a column at a specific flow rate (expressed in mL/min). Normal flow rates in HPLC are in the range of 1–2 mL/min. Pump can deliver either an isocratic or gradient mobile phase. The pump should have the following characteristics:

1. It should be non-corrosive.
2. It should have constant flow rate.
3. It should be compatible with mobile phase.
4. It should have the ability to bear the applied pressure for the flow of mobile phase.

Whereas the following factors may affect the efficacy (notably retention time, reproducibility, and detector sensitivity) of the pump:

1. Detector compatibility.
2. Viscosity.
3. Boiling point.
4. Flammability.

14.5.2.1 Types Pumps

There are two main types of pumps that are used in HPLC technique:

1. *Constant pressure pumps*: This type of pumps has some limitations such as limited reservoir and it allows non-pulsating flow. The example is displacement type pumps. It provides the continuous flow rate of sample by using the gas which is present in the cylinder.
2. *Constant volume pumps*: This type of pumps allow the flow of mobile phase into the column under high pressure. The examples are reciprocating type pumps, pressure vessel pumps, and syringe type pumps.

14.5.3 Sample Injector

Injector is used in HPLC for the introduction of the liquid sample. Care must be taken when sample is injected, it must not disturb the column packing. In this way, the liquid sample is directly entered into the flow stream of mobile phase in column. Injector is of two types that can be used in HPLC. One is single injection and other is an automated injection system. The injector must have the ability to withstand the high pressure that may be developed in the liquid system. Typical sample volumes of 5–20 μL can be injected.

14.5.3.1 Manual Injector

There are many components of the manual injector. It is a device that controlled the metered amount of the sample liquid and holds it prior to the injection. A valve which is used to maintain the path of the sample when there is direct injection of the sample liquid into the eluent stream. The injection is placed after the pump head. The sample is introduced in the load mode with the help of microsyringe under the high controlled conditions. The solution which is injected is stored in the sample loop. The injection valve is used in the sampling system in order to introduce the calculated amount of the sample and without changing of pressure of sample. When the knob is rotated to switch on the injection mode then sample is flow out from the loop. The sample is entered into the eluent stream. A schematic representation of operating systems of manual sample injector system has been described in Fig. 14.2.

14.5.3.2 Autosampler

Injection by an autosampler usually consists of the automatic execution of the operations. This can be done by using a microsyringe. This principle is based on a computer-controlled program. This operation is performed manually by the analyst with the help of machine. This type of sampling has the advantage that a large amount of the sample is injected.

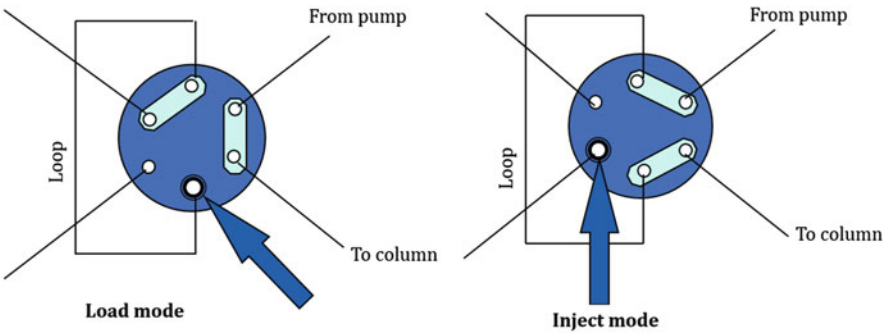

Fig. 14.2 Schematic representation of operating system of manual sample injector

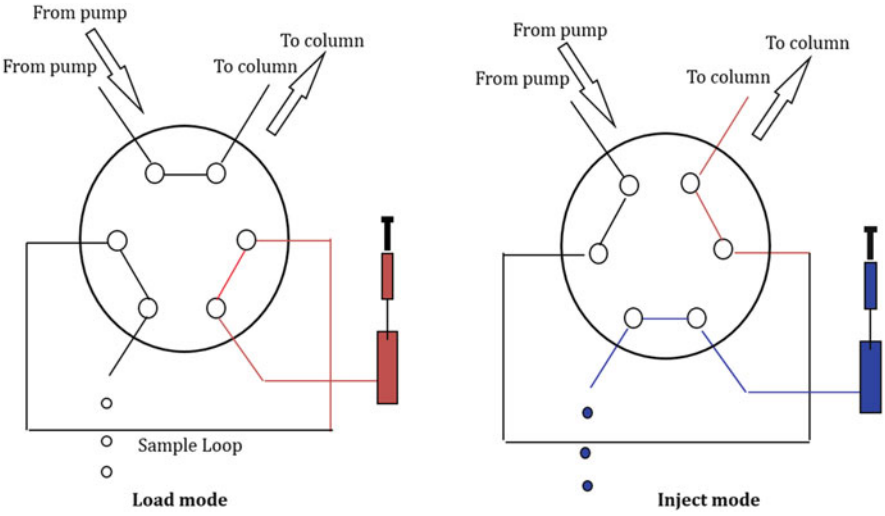

Fig. 14.3 Schematic representation of pressure injection method for sample injection into the column

Pressure Injection Method

With this method, after a specific amount of sample is aspirated from the sample vial and conveyed to the sample loop attached to a 6-port valve, the valve is switched so that eluent is delivered into the loop and the sample is consequently conveyed to the column. In other words, some of the operations performed by an analyst using a manual injector are performed by a machine. A schematic representation of pressure injection method for sample injection into the column has been described in Fig. 14.3.

Total-Volume Injection Method

In this method, a predetermined amount of the sample is introduced from the sample vial into a tube. The eluent is directly transferred into this tube. As a result, the sample is transferred into the column. The main advantage of this method is eluent flows inside the needle but not during injection operation. Another main advantage is that it can aspirate a volume greater than that conveyed to the column and there is a very little loss of sample. This may be impossible to aspirate the accurate amount of the sample if there are small air bubbles in the passage of sample from the measuring pump into the column needle tip. To avoid this situation, the fluid must be degassed that is filled. A schematic diagram of total-volume injection method for sample injection into the column has been described in Fig. 14.4.

14.5.4 Column

Columns are usually made of polished stainless steel and/or heavy glass materials. The reason to use stainless steel or glass material is to withstand the high pressure. Columns are usually narrow tubes that are packed with 25 μm particles. The column internal portion should be uniform and smooth. The average internal diameter of the column should be 4.6 mm, whereas the average length of the column should be 10–25 cm long and their internal diameter should be between 2 and 5 mm. The columns are most commonly filled with a stationary phase. For porous packing, silica is most commonly used with a particle size of 3–10 μm whereas, for pellicular packing, non-porous glass or polymer beads with 30–40 μm diameter are used.

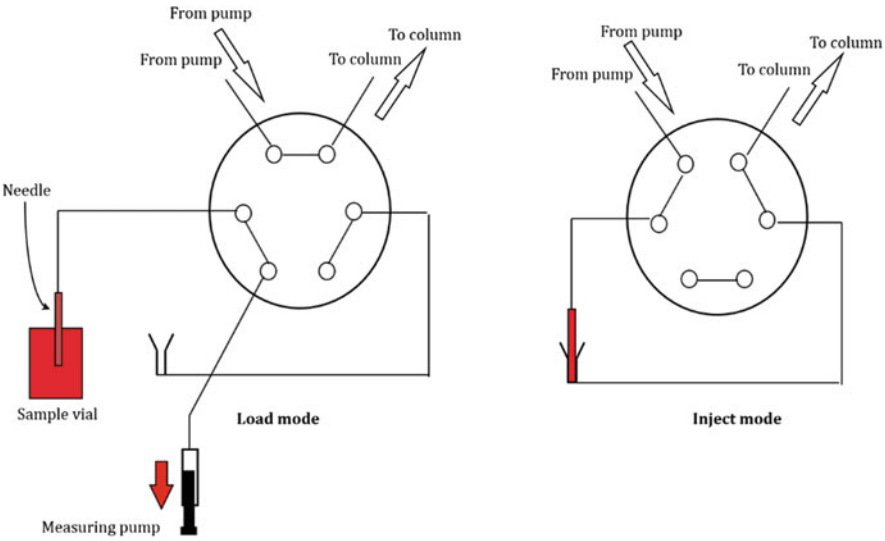

Fig. 14.4 Schematic representation of total-volume injection method for sample injection into the column

Columns having the internal diameters approximately less than 2 mm are mostly referred to use as microbore columns. The temperature of the mobile phase and the column phase should be kept constant during the whole experiment. For HPLC techniques, the most commonly used columns are as follows:

1. *Analytical columns*: These types of columns are used for quantitative analysis of the samples and they are often used in combination with UV-VIS detectors.
2. *Preparative columns*: These types of columns are used for the individual fractions of the components present in the sample mixture.
3. *Narrow bar columns*: These columns are 1–2 mm in length and are used when more sensitivity for the separation of the component is desired. These columns are used in combination with UV-VIS, fluoresce, and/or MS detectors.
4. *Capillary columns*: These columns are 0.3 mm in length and are made up of fused silica capillaries. They are exclusively used with alternative detection methods such as MS detectors.
5. *Guard columns*: These columns are short in length and are used to increase the life of the columns. These columns are placed between the sample injector and column and protect the column from the loss of efficiency and damage caused by particular matter and/or strongly adsorbed substance in the sample or mobile phase.

14.5.5 Detector

The detector can detect the individual components that come out from the column so that the analyst can quantitatively analyze the components of interest that have been separated from the sample. Detector then provides an output to the recorder or computer that interprets the results in the form of chromatogram. The most commonly used detectors in HPLC are as follows:

1. UV/Visible detector.
2. Photodiode array detector.
3. Refractive index detector.
4. Fluorescence detector.
5. Evaporative light scattering detector.
6. Amperometric detector.
7. Potentiometric detector.
8. Electrochemical detector.

14.5.6 Computer System

The computer system not only controls the overall modules of HPLC system but it also receives the signals from the detector and uses it to determine the time of elution (retention time) of the sample components for qualitative analysis and the amount of sample for quantitative analysis.

14.6 Factors Affecting on HPLC

Following are the most important factors that affect the efficiency of HPLC technique:

1. Particle size of the stationary phase packed in column.
2. Thickness of the stationary phase.
3. Length of the column.
4. Internal diameter of the column.
5. Flow rate of the mobile phase.
6. Nature of the mobile phase.
7. Viscosity of the mobile phase.
8. Affinity of the component of interest (analyte) with mobile phase and stationary phase.
9. Column packing.
10. Sample size.
11. Pump pressure.
12. Retention time.
13. Injection volume of sample.
14. Method of sample injection.

14.7 Advantages

1. Highly sensitive technique.
2. High performance.
3. Rapid process and hence time saving.
4. Can be used for qualitative as well as quantitative estimation.
5. Can be used for both analytical and preparative purpose.

14.8 Disadvantages

1. Irreversibly adsorbed components of the sample mixture cannot be detected.
2. Complexity may occur.
3. Costly technique.
4. Sometimes, the sensitivity of some specific compounds is low.
5. Co-elution of some compounds is difficult to detect.

14.9 Applications

1. It is used in the separation of inorganic ions, e.g., fluoride, chloride, bromide, phosphate, nitrite, magnesium, lead, copper, cadmium, zinc ions.
2. It is used for the detection of intoxicants and poisons in human blood.

3. It is used for the detection of addictive drugs, e.g., cocaine, heroin, morphine, alcohol, opioid drugs.
4. It is used for the separation of alkaloids present in plants.
5. It is used for the identification of illicit drugs.
6. It is exclusively used in forensic science for the investigation purpose.
7. It is used for the analysis of explosives.
8. It is used for the identification of lipids.
9. It is used for the identification of steroid hormones.
10. It is used for the separation and identification of bile acids.
11. It is exclusively used in nucleic acid research.
12. It is used in metabolic profiling.
13. It is exclusively used in the preparation, separation, and identification of natural products.
14. It is exclusively used in the analysis of pharmaceutical products.
15. It is used in the separation and identification of antibiotics.
16. It is used for the separation and identification of plant-based bioactive compounds.

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Gas Chromatography

15

Abstract

In this technique, the separation of the components present in mixture is based on the partition between the gaseous mobile phase and liquid stationary phase. In this chapter, types of gas chromatography are given. The advantages and disadvantages have also been described. The instrumentation of gas chromatography along with its working has been described. The factors that affect the GC have also been discussed. At the end of this chapter, advantages and disadvantages along with its application have been given.

Keywords

Principle of gas chromatography · Components of gas chromatography

15.1 Introduction

This chromatographic technique was invented by Martin in which he suggested that liquid mobile phase which is used in liquid chromatography can be replaced by using a suitable gas as mobile phase. As he used gaseous mobile phase instead of liquid mobile phase for separation purpose of the components of the sample, therefore, the term was named as gas chromatography. It is a process of separation of the components of the given sample such as crude substances by using a gaseous mobile phase. The more classy form of gas chromatography was developed by James and Martin in 1955. In gas chromatography, the mobile is in gaseous form, whereas the stationary phase is in solid or liquid form. If the component is more soluble in stationary phase, then it travels slower in the column, whereas if the component is less soluble in stationary phase, then it travels faster. Therefore, the components present in the sample mixture are separated according to their partition co-efficient between the component of interest and stationary phase.

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15.2 Principle

In this technique, the sample is first vaporized by heating and then it is injected into the head of the chromatographic column. The sample is transferred into the column by the flow of inert gaseous mobile phase. The column has a liquid stationary phase which is adsorbed on the surface of an inert solid. It has same principle as chromatography, separation of the components due to partition between stationary phase and mobile phase.

15.3 Types of Gas Chromatography

Based on the nature of the stationary phase, gas chromatography has the following two main types:

15.3.1 Gas–Solid Chromatography

When the stationary phase (adsorbent) is solid in nature, then it is known as gas–solid chromatography (GSC). The most common examples of stationary phase used in GSC are active carbon, silica, alumina, etc. The principle of separation in GSC is adsorption. One of the main advantages of GSC is that the column life is long, while the main disadvantage of this technique is that there may be chances of catalytic changes in the chemical composition of the components present in sample mixture.

15.3.2 Gas–Liquid Chromatography

If the stationary phase is liquid in the gas chromatography, then it is referred as gas–liquid chromatography (GLC). The solid surface which may be polymer is coated with the immobilized liquid. The principle in GLC is partition. Nowadays, GLC is abundantly utilized in the form of capillary column.

15.3.2.1 Advantages of GLC

1. It provides high resolution capacity for the complex mixtures. For example, separation of methyl esters of fatty acids.
2. As capillary column is abundantly used in GLC, only few microliter samples are enough for the complete analysis.
3. The seed of analysis is quite fast because the mobile phase has the ability to attain the rapid equilibrium between the mobile phase and stationary phase.
4. Sensitivity of detection is quite high while using different types of detectors.
5. It simultaneously allows qualitative and quantitative analysis of analyte.

15.3.2.2 Disadvantages of GLC

The major disadvantage of GLC is that the immobilized liquid is slowly run out from the solid surface.

15.4 Components of Gas Chromatography

The main components of gas chromatography have been illustrated in Fig. 15.1.

15.4.1 Carrier Gas

The carrier gas must be chemically inert. The most common gases that are used include nitrogen, helium, argon, and carbon dioxide. Hydrogen has better thermal conductivity but it also has disadvantage that it often reacts with the unsaturated compounds and compounds that are inflammable in nature. Thermal conductivity of helium is excellent but it is too much expensive. Nitrogen is inexpensive but it has reduced sensitivity. The selection of carrier gas mostly depends upon the type of detector which is used. The carrier gas system also contains a molecular sieve to remove water and other impurities. Following characteristics should be present in carrier gas:

1. It should be chemically inert in nature.
2. It should be suitable for the detector and must enable the detector to respond in a sufficient adequate manner.

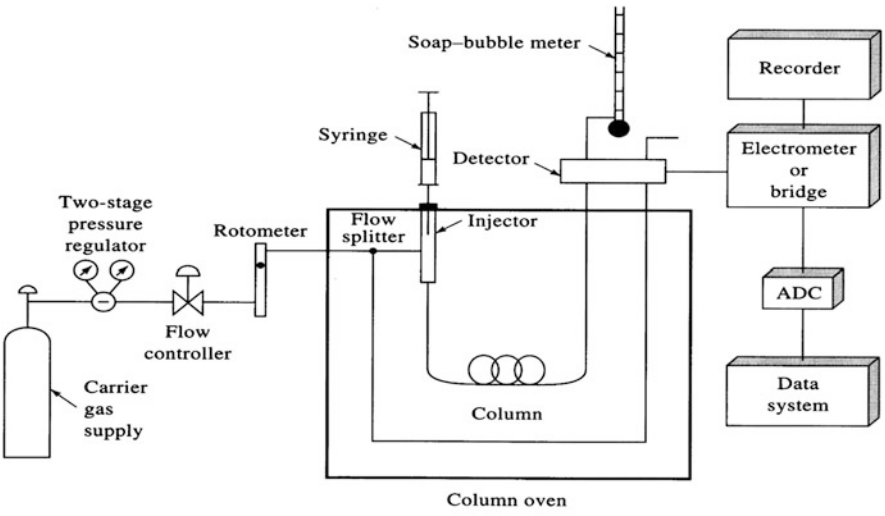

Fig. 15.1 Schematic representation of components of gas chromatography

3. The carrier gas should have the high purity.
4. It should be easily available.
5. It should be cheap.
6. It should be non-inflammable.
7. It is responsible for giving the best column performance.
8. It should be free from metallic particles.

15.4.2 Columns

The fundamental role of column is to separate the individual components present in sample mixture. There are two main types of columns that are used in gas chromatography technique.

15.4.2.1 Packed Columns

These columns contain glass or metallic tubes that are coated with immobilized liquid stationary phase. Most packed columns are 1.5–10 m in length and have an internal diameter of 2–4 mm.

15.4.2.2 Capillary Columns

These columns have very small internal diameter in millimeters but their length is between 25 and 60 m. These capillary columns have immobilized stationary phase which is liquid. The stationary phase is coated with the glass or silica in the inner sides of columns. These are also called as open tubular columns and have the following types:

1. *Wall-coated open tubular columns:* The inner walls of these columns are coated with inert active material which acts as a support on which the immobilized liquid stationary phase is adsorbed. Therefore, these columns are also known as support-coated open tubular columns.
2. *Support-coated open tubular columns*: Support-coated open tubular columns are usually coated with layer of the support material having micron size. This layer is further coated by the thin film of immobilized liquid stationary phase. These types of columns usually have more capacity for sample as compared to that of wall-coated open tubular columns.

15.4.2.3 Factors Affecting on Column Efficacy

Following are the important factors that may influence the overall efficacy of the column:

1. *Column breakage*: This column may break due to many reasons such as if coating of the column is done by the weak coating material or may be coating is not done properly. Due to variation in the temperature of the column which may increase or decrease depending upon the conditions is also a reason for column breakage. If the diameter of the column is large then breakage may occur due to this reason.

2. *Thermal damage*: Higher temperature of the column during heating may also cause the degradation of stationary phase. Due to presence of oxygen during the whole operation thermal degradation may be increased. Similarly, the presence of chemical compounds, such as non-volatile compounds, acids (HCl, H_2SO_4, HNO_3), bases (KOH, NaOH), organic compounds (perfluoro acids), may also damage the column.
3. *Column contamination*: It may be either because of the non-volatile or semi-volatile contaminants.

15.4.3 Temperature Programmer

The column of GLC is interposed in the thermostatic oven. It is temperature-controlled device which monitors and regulates the overall temperature of the column. It is recommended to use thermostatically controlled oven for efficient separation. Sometimes, preheaters are also used to convert the sample into its vapor form. These preheaters are present along with injecting devices. There are two main types of temperature programmers which as follows:

1. *Isothermal programming*: During the whole experiment, the temperature is kept constant.
2. *Gradient programming*: During the whole experiment, the temperature varies. In this case temperature may be increased or decreased depending upon the conditions.

15.4.3.1 Factors Affecting on Temperature Programming

During the operation of GC, temperature programming should be performed. The factors that affect the temperature programming are of the following:

1. *Flow rate of the mobile phase*: It affects the elution rates of the components of interest present in sample. When the flow rate of mobile phase in the column is high, then components remain for the shorter period of time in column as a result broadening the peak.
2. *Stability of the stationary phase*: If the sample shows the solubility within stationary phase, then residential time of sample in column is increased and better results can be achieved.
3. *Stability and solubility of analyte*: Temperature affects the stability and solubility of analyte. If the temperature increases, the solubility of the gas in liquid decreases and it reduces the retention time of the sample in the column.
4. *Volatility of analyte*: If the sample evaporates rapidly by applying the heat, then components of the sample are eluted rapidly.

15.4.4 Sample Injector

The sample is injected into the carrier gas flow to the column of HPLC by using sample injector. For the analysis of the gaseous samples, mostly rotary valve is used. This rotary valve is mostly used in gas chromatography but volume of sample is kept small than used in HPLC. In the case of the liquid sample, the sample is introduced into the heated lash by using the gas syringe. The sample evaporated and the carrier gas is responsible for the transferring of the vaporized sample from the injection site to the column. In the case of the solid samples, the sample is initially heated at a high temperature for the volatilization of the sample. As a result, the sample is converted into the volatile derivatives substances.

15.4.5 Detectors

There are many types of detectors which can be used in gas chromatography. Detectors can be grouped into the following two main types:

1. *Concentration-dependent detectors*: The signal detected by a concentration-dependent detector is directly related to the amount of solute in the detector. This type of detector cannot cause the destruction of the sample. The detector response may be lower due to dilution with make-up gas.
2. *Mass flow-dependent detectors*: This type of detectors mostly destroy the sample. The rate at which solute components enter into the detector affects the signals. The make-up gas has no influence on the efficiency of mass flow-dependent detectors.

Different kinds of detectors exhibit the different level of selectivity. The non-selective type of detectors can respond to all types of the components of the sample mixture except for the carrier gas. The selective type of detectors responds to a wide range of compounds having common physical characteristics. Wide range of detectors are used in gas chromatography, the most commonly used detectors are as follows:

1. *Flame ionization detector (FID)*: It is highly sensitive detector. Two different kinds of gases are placed in the cylinder, which are used for the ignition of the flame. The effluent of the column is directed towards the flame and the potential difference is detected.
2. *Thermal conductivity detector (TCD)*: This detector contains the heated filament. When the carrier gas is passed through the cell, change in the filament current which is compared with the reference cell. Due to difference in current a signal is produced.
3. *Electron capture detector (ECD)*: It specifically detects the halogen containing compound. The sample is ionized due to radioactive material. This ionization current is quenched by the compounds containing halogen compounds.

4. *Nitrogen-phosphorus detector*: This is used for the detection of the compounds having nitrogen and phosphorus. Its principle is same as flame ionization detectors.
5. *Flame photometric detector (FPD)*: It is used for the detection of the sulfur and phosphorus. The eluent is passed through the flame as a result excited species are formed and the light is produced in the flame.
6. *Photo-ionization detector (PID)*: This type of detector is used for the detection of the aromatic compounds. The eluted molecules are photoionized by the ultraviolet radiation. The compounds that have low ionization energy are detected.

15.4.6 Recorder and Read-Out Device

Signals for separated fractions of vaporized components, received from the detectors are recorded by recorder and read-out device interpret the input responses received from detector into results.

15.4.6.1 Features of Detectors

Following are the ideal characteristics of the detector:

1. It should be easily handled easily.
2. The decomposition of the sample may not occur due to the detectors.
3. It can measure the wide range of temperature.
4. The sensitivity and reproducibility of the detectors must be high.
5. The detectors must have high stability.
6. It should not produce noise.
7. Small volume of the sample can be used in order to avoid peak broadening.

15.5 Factors Affecting on Gas Chromatography

1. *Volatility of compounds*: The components of the sample that have low boiling point will travel faster than the compounds having high boiling components through the column.
2. *Polarity of the compounds*: If the polar column is used, then the components of the sample that are polar in nature will move more slowly and vice versa.
3. *Column temperature*: If the temperature is increased, then the components of the sample will eluted very rapidly.
4. *Column packing polarity*: All the components of the compounds will move slower in polar column as compared to that of non-polar column.
5. *Flow rate of mobile phase*: Speeding up the flow rate of mobile phase also increases the speed of all compounds to be moved with mobile phase.
6. *Length of column*: If the column have longer length then it will take longer time for elution of all the components present in mixture and as a result better will be the separation.

15.6 Advantages

1. This technique has strong power for the separation of even complex mixture that can be resolved into its constituents.
2. The method is highly sensitive.
3. Small sample is required for analysis.
4. It contains high sensitivity detector system.
5. It has good precision and accuracy.
6. The operation is completed in a very short interval of time.
7. Linearity is good.
8. The instrument cost is relatively low.
9. It has generally longer life.
10. The technique is relatively suitable for routine analysis.
11. Precision is high.
12. Easy to handle the equipment.

15.7 Disadvantages

1. Sensitivity is low.
2. Volatilization is required for the analysis and there may be a chance for the degradation of the sample.
3. It cannot be used for analysis of biological sample because of the high temperature of the column.

15.8 Applications

1. It is widely used for analysis of gaseous samples.
2. It is used for the estimation of amount of CO_2 present in the fuel gases.
3. It is used for the estimation of organometallics.
4. It is used for the estrogens analysis.
5. In pharmaceutical industry, it is widely used for the analysis of the following drugs:
   a. Anti-tuberculosis drugs.
   b. Antibiotics.
   c. Antiviral drugs.
   d. Anti-neoplastic agents.
   e. Ointments.
   f. Anticonvulsants.
   g. Steroids.
6. In food industry, it is also used for the analysis of dairy products.
7. It is used for the determination of pesticides in aquaculture products.
8. It is used for the diagnosis of certain diseases like cancer.
9. It is used for the analysis of plant-based bioactive compounds and essential oils.
10. It is used for the detection of narcotics and alcohols in blood.

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Introduction to Thermal Analysis

16

Abstract

Thermal analysis is an analytical technique in which characteristics of the materials which involves the temperature and/or heat are studied. Usually, the measurements are made either by increasing or decreasing the temperature. Broadly speaking, any measuring and/or analytical technique can be made as thermal analysis technique which involves the temperature and/or heat as a key function for analysis. In this chapter, we have briefly introduced thermal analysis and its basic principle. Moreover, we have also discussed the types and applications of thermal analysis.

Keywords

Types of thermal analysis · Principle of thermal analysis · Applications of thermal analysis

16.1 Introduction

Thermal analysis is a branch of physicochemical science where the properties of materials are studied and quantified as they change with respect to the change in temperature and/or heat. According to International Union of Pure and Applied Chemistry, thermal analysis is defined as a group of techniques in which a physicochemical property of the material is measured as the function of temperature, while the sample is subjected to a controlled temperature programs like cooling, heating, and/or isothermal process. Thermal analysis is basically a series of techniques that are used to study the characterization and/or properties of material with respect to change in temperature and/or heat. Thermal analysis is basically used to study the physical properties of the material such as mass, enthalpy, dimension, dynamic characteristics, freezing temperature, boiling temperature, melting temperature, curing rates for adhesives, heat of fusion, heat of vaporization, etc. Thermal analysis cannot be used for structure analysis of a compound. Thermal analysis is widely used

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in different disciplines notably from pharmaceutical sciences to polymer sciences and materials chemistry where the changes in the behavior of sample with respect to basic characteristics of materials along with its wide range of applications in academia and quality control in industry, in research and development are monitored under temperature-controlled conditions.

16.2 Principle

The basic principle in all types of thermal analysis techniques is the same. To study a sample, its reference is also used. Both the reference and sample are heated at an identical temperature, even when a thermal event occurs in the sample. The energy required to obtain a zero temperature is measured precisely. Following are the most important components of thermal analysis that are mandatory for the basic principle of thermal analysis:

1. Sample holder and/or compartment to hold the sample and/or reference during thermal analysis. In modern equipment, mostly two compartments/pans are present. One for the reference material and the other one for the sample or analyte. Sample holders are mostly made up of platinum and/or aluminum.
2. A heater on which the pans are placed. The heaters are attached with a computer whose function is to switch on the heaters and let them to heat at a specific rate. Computer makes sure that heating rate remains the same throughout the process.
3. Sensors to measure the property of the sample and/or note the temperature difference between the reference and sample material. There are separate sensors for the reference and for the sample. The sensors used are mostly platinum resistance thermocouples.
4. An enclosure and/or insulator within which the experimental parameters are controlled.
5. Read-out device to record the data collection and processing. The recorder presents the result in the graphical representation in the form of calibration curve.

16.3 Types of Thermal Analysis

Depending upon the physical properties of the material to be measured, the most commonly used thermal analytical techniques are as follows:

1. Differential Scanning Calorimetry (DSC): It measures the heat which is being absorbed or released during the process of heating or cooling. DSC is used to measure the heat of reaction, melting point, heat capacity, and glass transition.
2. Differential Thermal Analysis (DTA): Used for thermal investigation or analysis where the thermal change can be studied and investigated. It is used to determine the oxidation process, decomposition, and loss of water or solvent.

3. Thermo Gravimetric Analysis (TGA): It measures the change in weight of the sample during the process of heating or cooling. It is used to measure the phase changes, glass transition, and melting point.
4. Thermo Mechanical Analysis (TMA): It measures the change in dimensions during the process of heating or cooling. It can be used to analyze the sample expansion, penetration, contraction, softening, and glass transition.

16.4 Applications

Thermal analysis is mainly used in the field of research and development but nowadays, common materials including pharmaceutical products, foods, polymers, ceramics, electronic materials, organic compounds, inorganic compounds, and even biological organisms can be studied through thermal analysis. Thermal analysis is being widely used as a testing standard in the following areas:

1. Quality control of pharmaceutical products.
2. In process quality control during the manufacturing of pharmaceutical products.
3. Inspection of raw materials that are used for the manufacturing of pharmaceutical products.
4. Determination of glass transition temperature of materials used for the manufacturing of pharmaceutical products.
5. Melting points determination.
6. Crystallization time and temperatures of polymers and polymeric materials that are used for the manufacturing of pharmaceutical products.
7. Heat of melting and crystallization of polymers and polymeric materials.
8. Determination of impurities in the polymers by examining thermograms and plasticizers can also be detected.
9. Oxidative stabilities of pharmaceutical products.
10. Compositional analysis of raw materials and pharmaceutical products.
11. Heat capacity determination.
12. Determination of impurities that may be present in polymers and polymeric materials.
13. Determination of compatibility of active pharmaceutical ingredients with that of excipients that are used for the manufacturing of pharmaceutical products.
14. Used to review the kinetics of solid state of API including decomposition, accelerated stability, and aging effect on various formulations.
15. Determination of nature polymorphism and/or crystallinity of the compound.
16. Physicochemical properties of the polymers, polymeric materials, excipients, and active pharmaceutical ingredients.
17. Used for accelerated stability studies.
18. For the analysis of the results of lyophilization.
19. Estimation of moisture contents during in process quality control of pharmaceutical products.
20. Determination of denaturation of proteins.

21. Determination of protein folding.
22. In food sciences to determine water dynamics as change in water distribution may be correlated with texture changes.
23. Along with X-ray diffraction and IR spectroscopy used for the screening of compatibility of drugs with excipients.

16.5 Advantages

1. Solid, semi-solid, or liquids samples can be analyzed
2. Small sample size is required only
3. Sample preparation is minimum
4. Quantitative analysis of multiple mass loss thermal events
5. Can separate and analyze multiple overlapping mass loss events

16.6 Disadvantages

1. Evolved products can be identified only evolved gas analyzer is connected.
2. Uncontrolled temperature can destroy the sample.

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Differential Scanning Calorimetry

17

Abstract

Differential scanning calorimetry (DSC) is a thermal analysis technique which involves the measurement of temperature difference between the sample and the reference material as a function of the temperature while sample and reference both are subjected to a controlled temperature program. This technique is especially used for qualitative and quantitative determination of change in temperature in terms of exothermic, endothermic, and heat capacity. In this chapter we have discussed the principle and types of DSC. The comprehensive instrumentation, working of the DSC, and the factors affecting the DSC curve have also been discussed in detail. The interpretation of the result through the curve is also elaborated. At the end of this chapter, advantages and disadvantages along with application have been described.

Keywords

Principle of DSC · Types of DSC · Sample preparation for DSC

17.1 Introduction

DSC is a thermo-analytical technique which involves the measurement of the difference in the amount of heat required to increase the temperature of the sample and reference as a function of temperature. Sample and reference both are maintained at almost the same temperature throughout the experiment. Calorimetry is the process of measuring the amount of heat released or absorbed during a chemical reaction and the device is known as calorimeter. Watson and O'Neill developed this thermal analysis in 1960 and then introduced it in 1963 commercially at Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy.

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17.2 Principle

The basic principle involved in DSC is that this technique is used to study what is the effect of heating on the polymers/samples. It examines the thermal transitions of polymer or sample when heated. For example, this technique can be employed to study the effect of heating on a crystalline polymer, glass transitions, and crystallization. The sample and reference materials are heated by separate heaters at the same temperature throughout the experiment. The energy which is required to obtain zero temperature difference between sample and reference is measure.

17.3 Types of Differential Scanning Calorimeter

There are two methods involved in DSC which have been described as follows:

17.3.1 Heat Flux DSC

It is a technique where temperature of sample and reference is changed in a specific program. The difference of the temperature between sample and reference is measured as a function of heat.

17.3.2 Power Compensated DSC

It is a technique where difference of thermal energy applied to sample per unit time is measured between the sample and reference as function of temperature.

17.4 Instrumentation and Working

The main components of DSC have been illustrated in Fig. 17.1. Two pans are used in DSC, one for the sample and other for reference. In sample pan, sample (polymer) is placed while the reference pan is kept empty as shown in Fig. 17.1. Each pan has a heater underneath which are connected with a computer which turns on heaters and heat the two pans at a specific rate, usually 10 °C per min. The computer is used to regulate a constant heating rate during the whole experiment. But the heaters do not provide the heat at a constant rate. That is because both pans are different, one contains polymer while other does not. Therefore, an extra heat is required for the sample pan to keep a zero temperature difference between the sample and reference. So, the heater underneath the sample pan has to do more work than the other heater. DSC experiment measures this extra heat of that heater under sample pan by plotting a graph in which x-axis corresponds to temperature and y-axis corresponds to difference in temperatures of both heaters.

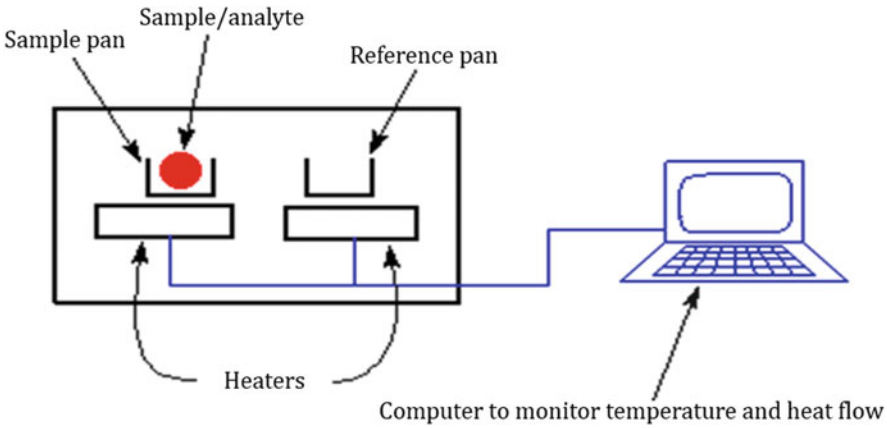

Fig. 17.1 Schematic representation of DSC apparatus

17.4.1 Sample Preparation for DSC Analysis

Accurately weighed amount of sample (usually ~3 to 20 mg) is placed in sample pan. Sample pan is usually made of inert or treated metals, e.g., aluminum, platinum, nickel, etc. In the DSC analysis, aluminum pans are mostly used if the sample does not react with Al and the temperature of sample do not exceed to 600 °C. Pans are made up of different kinds of material, e.g., hermetically sealed (airtight) pans, pinhole, or open pans. It is very important that for a single experiment, the sample and the reference pan should made up of same kind of material. It is also important that the material should be placed to cover the bottom of the pan completely to ensure good thermal contact. It is recommended to avoid overfilling of the pan to reduce the thermal lag from the bulk of the sample to the sensor. This may be happened because resolution increases due to sample having less masses and low heating rates.

17.4.2 Purge Gases

Sample may react with air and oxidize or burn. This problem is overcome by using inert gases. Most commonly used inert gases are nitrogen, helium, argon, etc. Nitrogen increases the sensitivity of the experiment. Its typical flow rate is 50 mL/min. Helium increases the resolution of the peaks and its flow rate is 25 mL/min. Sometimes, the air and/or oxygen are deliberately used to analyze the oxidative effects of the sample. In that case the flow rate of oxygen is 50 mL/min.

17.4.3 Heating Rate

It is an important factor that may influence the accuracy of the results. Faster heating rate may increase the sensitivity but it will decrease the resolution. While, slow heating rate may decrease the sensitivity but it will increase the resolution. Therefore, the ideal heating rate is 10 °C/min in which the accuracy and sensitivity do not change.

17.5 Calibration of DSC

It depends upon the estimation of the thermal resistance of both the sample and reference sensors and the measurements which are made over the interested temperature range. For the calibration of DSC, firstly the temperature difference is measured between the two empty pans and then thermal response is determined for the sample material Because DSC is used to measure the difference in heat flow between a sample pan and reference pan. If there is a difference in heat capacity of the sample and reference, then the baseline stabilizes faster. This is retained small by adding more weight (same material on pan) to the reference pan so that it is similar in total weight to the sample pan.

17.6 DSC Curves

The result of a DSC experiment is displayed in the form of a graph or a curve which is known as DSC curve. This graph is plotted between heat flux and temperature or time. There are two different conventions, exothermic and endothermic. If exothermic reactions take place then the sample shows a positive peak or negative peak. This peak depends upon the type of technology used in the experiment. This curve is used to measure the enthalpies of transitions. This can be measured by integrating the peak corresponding to a given transition. The enthalpy of transition can be expressed by using equation:

$$\Delta H = KA.$$

17.6.1 Factors Affecting DSC Curve

Following are the main factors that may influence the overall results of the DSC analysis:

17.6.1.1 Instrumental Factors

Following are the important types of instrumental factors that may affect the results of DSC:

1. Furnace heating rate: Heating rate should be in the range of 5–10 °C/min.
2. Recording or chart speed.
3. Furnace atmosphere: Samples are normally put under an inert gas usually N_2 or He, in order to avoid the oxidation or corrosion.
4. Composition of the sample holder.
5. Sensors location.
6. Sensitivity and accuracy of the recoding system.

17.6.1.2 Sample Characteristics

Sample is also an important factor that may also influence the results of DSC. Here are the main factors of the sample that may influence on results of DSC:

1. Amount of sample: In order to get accurate results, the sample mass should be in the range of 3–15 mg, because different amount can give rise different results.
2. Sample nature.
3. Sample preparation.
4. Solubility of evolved gases in the sample.
5. Particle size and shape of the sample.
6. Thermal conductivity.
7. Heat of reaction.

17.7 Advantages

1. A small amount of sample can be used in this technique.
2. The sample present in any form can be tested.
3. It does not require more time for completion.
4. It can be used for studying many types of reactions.
5. It does not require calibration for the entire range of temperature.

17.8 Disadvantages

1. It is not suitable for two-phase mixture.
2. It does not detect the gas generation.
3. There is uncertainty in determined values of heat of transfusion and transition temperature.
4. It can be used for thermal screening of isolated products and intermediates.
5. The test sample containing solvent may be volatile. So, it is very difficult to prepare test sample.
6. Interpretation of result is very difficult.
7. It cannot optimize the sensitivity and resolution in a single experiment.

17.9 Applications

1. *Study of liquid crystals*: Some materials can change their state from solid to liquid and then pass through a third state anisotropic state, which exhibit the properties of both solid and liquid phases. This state is called as a liquid crystalline state or mesomorphous state. DSC can be used to observe these changes in all these states.
2. *Study of oxidative stability*: An airtight sample chamber is used to study the stability to oxidation of samples. By altering the atmosphere of the sample, the test is done isothermally. First, the sample temperature is set at the desired temperature under an inert atmosphere that is achieved by inserting the nitrogen gas. Then, oxygen gas is inserted into the system to achieve oxidation. The oxidation is observed as a deviation in the baseline. The study of oxidation process helps in estimating the stability and determining the optimum storage conditions for a drug compound.
3. *Study of drug analysis*: Used to study tablet coating. DSC is used to determine moisture content in drug. DSC is used to study solid drug dispersion. DSC is used to determine the drying temperature of different excipients. DSC with support of X-ray diffraction is used as screening technique for the compatibility testing of drug with excipient. DSC is a valuable tool in choice of suppository base.
4. *Study of chemical analysis*: DSC can be used to monitor melting point depression. This is possible because the temperature range over is dependent on the relative amounts of the sample. Consequently, less pure compounds will exhibit a broadened melting dip that begins at lower temperature than a pure compound. Freezing point depression can be used as a purity analysis tool. This is possible because the temperature range over which a mixture melt is dependent on their relative amount. Less pure compounds will exhibit a broadened melting dip that begins at lower temperature than a pure compound.
5. *Study of polymer characteristics*: DSC is also used for analyzing polymers to check their composition. Melting points and glass transition temperatures for most polymers are available from standard compilations, and the method can show up possible polymer degradation by the lowering of the expected melting point, which depends on the molecular weight of the polymer, so lower grades will have lower melting points than the expected. Impurities in polymers can be determined by examining thermograms for anomalous peaks, and plasticizers can be detected at their characteristic boiling points.
6. *Study of food analysis*: In food sciences, DSC and any other thermal analytical techniques are used in combination to analyze the water dynamics.
7. *Study of crystallization phenomenon*: DSC can also tell us about the crystallinity nature of a polymer. If we know the latent heat of melting, ΔH_m, we can figure out the answer. The first thing we have to do is to measure the peak area for the melting of a polymer.
8. *Determination of heat capacity*: It can be used for the determination of heat capacity. When a certain amount of heat is transferred to the sample, its

temperature increases by a certain amount, and the amount of heat it takes to get a certain temperature increase is called the heat capacity, or Cp, it can be figured up from the DSC plot.

9. *Determination of glass transition temperature (Tg)*: Tg is an important characteristic of non-crystalline and semi-crystalline materials, but Tg is a particularly significant property of many common polymers. At a temperature below Tg, amorphous and semi-crystalline polymers tend to be hard and brittle because the polymer chains are locked in a tangled, coiled position. Above Tg, the polymeric chains are able to more easily rotate and slip past each other, and the polymer becomes softer and more ductile.
10. *Study of proteins*: DSC is used to characterize the stability of a protein. It does this by measuring the heat changes associated with the molecule's thermal degradation when heated at constant rate.
11. *Qualitative and quantitative analysis mineral*: DSC is used for detection of any mineral in a sample.
12. *Study of antibody domains*: DSC use to study antibody domains.
13. *Study in drug development*: DSC is used to determine the status of any process during the drug development.
14. *Study of enzyme kinetics*: DSC is used to study the reaction time of enzyme degradation and/or kinetics.
15. *Binding studies*: DSC is a valuable approach for studying binding between a biological macromolecule and a ligand such as another polymer.

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Differential Thermal Analysis

18

Abstract

Differential thermal analysis (DTA) is a thermo-analytical technique which is used for thermal analysis where thermal changes can be studied. It is used to determine the oxidation process, decomposition, and loss of water or solvent. In this chapter we have discussed the basic principle of DTA along with its instrumentation, working, factors affecting DTA curve, advantages and disadvantages, and its applications.

Keywords

Principle of DTA · Instrumentation of DTA · Working of DTA

18.1 Introduction

DTA is a type of thermo-analytical technique. Robert Austen was the first scientist who worked on this technique by introducing two thermocouples in its apparatus in 1899. Then, in 1909, Burgess modified this technique followed by Norton (1939), Kerr (1948), Kauffman (1950), Grim (1951), and Fold Vari (1958). However, differential thermocouple arrangement which was suggested by Robert-A is still widely used in thermal analysis and therefore this technique is known as DTA or "Thermography." In DTA, the temperature difference is measured between the sample/analyte and the reference as a function of temperature while the sample and the reference are heated at a controlled temperature program. A plot (curve or thermogram) is then plotted between the differential temperature and time or temperature. Then changes observed in the sample, due to the absorption or evolution of heat, are detected relative to the reference.

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18.2 Principle

DTA is a thermal analysis which involves the comparison between the temperatures of sample under investigation and a thermally inert material/reference which may be α-alumina (it is densified alumina in α-phase and occurs in hexagonal structures) and this comparison is then recorded with the furnace temperature as the sample is heated or cooled at uniform rate. There is a constant temperature difference (ΔT) between the sample and the reference as they both have different heat capacities. But when the sample undergoes the endothermic or exothermic changes then ΔT becomes different. The basic principle involves the measurement in temperature changes which are associated with the physical changes or chemical changes of the sample during the gradual heating process.

18.3 Instrumentation

DTA contains the following basic components. Schematic representation of overall equipment of DTA has been illustrated in Figs. 18.1 and 18.2.

18.3.1 Furnace Assembly

It works as a temperature programmer. There are many furnaces which are used depending upon the sample material and the rate of heating, e.g., nichrome furnace which is made up of Nickel and Chromium alloy is used when rate of heating is up to 1300 °C. Platinum furnace which is made of Pt and its alloys is employed when rate of heating is up to 1750 °C and the furnace which is made of molybdenum is employed for higher temperature ranges that are up to 2000 °C.

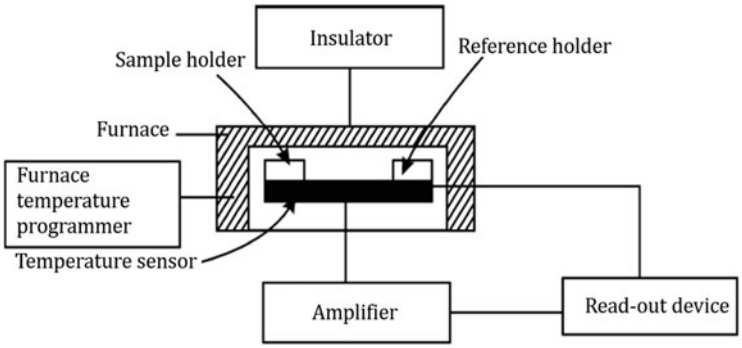

Fig. 18.1 Schematic representation of components of DTA

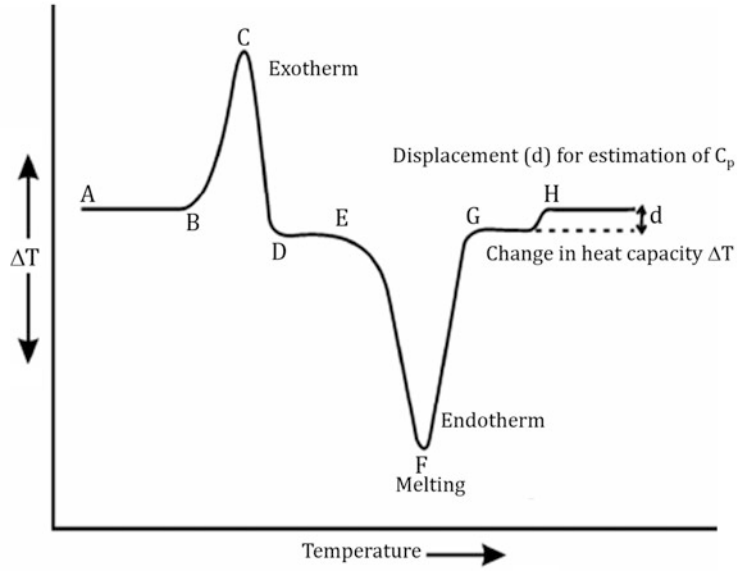

Fig. 18.2 Schematic representation of characteristics of DTA

18.3.2 Sample and Reference Holder with Temperature Detector

In DTA apparatus, two compartments/pans are present, one for the sample and other one for the reference. The holders are designed in a manner that they can accommodate even a small quantity of the sample or reference material and give maximum thermal effect. Mostly, they are made up of platinum, stainless steel, nickel, silver, and alloys such as platinum-rhodium. Many ceramic materials, e.g., silica, sintered alumina, fire clay, heat resistant glass, and graphite are recommended as holders for the sample and reference (like α-alumina). The holders are connected with temperature detector which measures the difference in temperatures of the sample and reference material as a function of time or temperature when the temperature is rising at the constant linear rate. In simple words, both pans are different, one is sample pan which loads polymer means extra material and other one is the reference does not has polymer. Due to extra material more heat is required to keep the sample pan's temperature increasing at a same rate as that of the reference pan.

18.3.3 Furnace Temperature Programmer

Electronic temperature regulators are used to ensure a constant rate of heating of the furnace. For temperature regulation, thermocouples made up of rare metal alloy such as platinum (Pt-10-13% Rh) are mostly employed for the measurements of temperature up to 2000 °C. A thin thermocouple is usually inserted in both holders. When few samples are to be studied, visual galvanometric observations are employed

though inconvenient. But for accurate results and convenience automatic pen and ink electronic recorder are used mostly.

18.3.4 Amplifier

Its function is to convert the heat signal into electrical signal.

18.3.5 Read-Out Device

It is used to display the results in the form of a thermogram. Nowadays, the read-out devices have microprocessors that deliver outputs compatible with computers and printers thus minimizing the risk of operator errors.

18.3.6 Insulator for Furnace and Sample Holder

It a block of ceramic or other insulating material enclosing the furnace and sample holders which does not readily allow the passage of heat.

18.4 Working of DTA

The sample/analyte under investigation is loaded into a container. This container is then placed onto the sample pan and it is marked as S (means sample). Same quantity of inert material (like alumina) as that of sample is placed in another container which is then placed onto the reference pan and it is marked as R (means reference). In order to heat the sample pan and the reference pan at an identical rate, the dimensions of these two pans should be nearly identical; moreover, the sample and the reference should have equal weights, thermally matched and should be arranged symmetrically with the furnace. The metal block which surrounds the pans acts as a heat sink whose temperature is increased slowly by using an internal heater. The sink then heats the sample and reference material simultaneously. Two pairs of thermocouples are used, one pair is in contact with the sample and the second pair is in contact with the reference. Thermocouple is attached with an amplifier which amplifies the result of differential thermocouple and sent this result to the read-out device which displays the results in the form of DTA curve or thermogram as a function of the sample temperature, reference temperature or time. No signal is generated if no temperature difference is observed even though the actual temperatures of both the sample and reference are increasing. When there is a physical change in the sample then heat is absorbed or released. For example, when a metal carbonate is decomposed then carbon dioxide is released. This is an endothermic reaction where the heat is absorbed and the temperature of the sample is decreased. Now the sample is at a

lower temperature than that of the reference. This temperature difference between sample and reference produces a net signal, which is then recorded.

18.5 Characteristics of DTA Curve

The DTA curve or thermogram is a plot between differential temperature (ΔT) and temperature of reference (T). DTA curve may be endothermic (downward plot) or exothermic (upward plot). In exothermic reaction, the sample temperature is higher than that of the reference, whereas in endothermic reactions, the sample temperature is less than that of the reference material and if there is no reaction happening in the sample material, then the sample temperature remains the same as that of the reference material. As shown in Fig. 18.2, the value of ΔT is zero along the line AB which indicates no reaction in the sample material. At point B the curve rises from the baseline due to the exothermic reaction and it forms a peak BCD with a maximum temperature or heat value at point C. At this point, the rate of evolution of heat is almost same to that of the difference between the heat evolution of the sample and the reference. However, it does not show the maximum rate of heat evolution or completeness of reaction. So, point C is not important in DTA curves. At point C, the process of heat is completed and after this, heat is going to be decreased up to D point. The peak temperature is the characteristic of the sample material. BCD area has a direct relation with the amount of reacting material. Identification of the sample material, heat of reactions, sample mass (m), heat of reactions (H) sample geometry, and thermal conductivity are the prospects which can be determined using DTA curves.

18.6 Factors Affecting on DTA Curve

Differential thermal analysis is not a dynamic thermal analytical technique. Its value can deviate because of many factors which can be divided into four major groups.

18.6.1 Sample Factors

1. Amount of the sample.
2. Packing density.
3. Particle size of the sample material.
4. Degree of crystallinity.
5. Heat capacity.
6. Thermal conductivity.
7. Dilutes of the diluents.
8. Swelling of the sample.
9. Shrinkage of the sample.

All these sample factors can affect the DTA curve or thermogram.

18.6.2 Instrumental Factors

1. Size or shape of the holders.
2. Material of the sample holder.
3. Recording system sensitivity.
4. Rate of heating of the sample.
5. Atmosphere around the sample.
6. Thermocouple location in the sample.
7. Instrumental design.

18.6.3 Physical Factors

They can be divided into two further groups. Some physical factors affect the exothermic curve or some affect the endothermic curve. Exo-thermogram may be affected by one of the following factors:

1. Adsorption.
2. Change in the crystal structure.
3. Crystallization.

Whereas endo-thermograms may be affected by one of the following factors:

1. Desorption.
2. Change in the crystal structure.
3. Melting.
4. Vaporization.
5. Sublimation.

18.6.4 Chemical Factors

They can also be divided into two further groups. Some chemical factors affect the exothermic curve or some affect the endothermic curve. Chemical factors which may affect the exothermic reactions are as follows:

1. Oxidation.
2. Breakdown reactions.
3. Chemisorption.
4. Solid state reactions.

Chemical factors which may affect the endothermic reactions are as follows:

1. Reduction.
2. Breakdown reactions.
3. Solid state reactions.

18.7 Advantages

1. DTA apparatus can be operated at very high temperature ranges.
2. Highly sensitive technique.
3. Both exothermic and endothermic reactions can be determined accurately.

18.8 Disadvantages

1. There is lot of uncertainty in transition reactions and heat of fusions.

18.9 Applications

1. Used to identify the minerals both qualitatively and quantitatively.
2. Polymers characteristics can be easily characterized.
3. Degree of crystallinity can be measured.
4. Degree of polymerization can be assessed.
5. Many of the biological materials can be analyzed.
6. Melting point, boiling point, and temperatures of decomposition of organic compounds can be determined.
7. Have wide applications for the quality control (QC) of many substances such as soil, cement, glass, etc.
8. Also used to determine the thermal stability of many inorganic compound s and complexes.

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Thermo Gravimetric Analysis

19

Abstract

In this thermo gravimetric analysis (TGA) technique, sample is heated at a given temperature with controlled heating rate to measure the change in the weight of a sample substance as a function of temperature. The results can be interpreted from TGA curve. Thermogram is plotted between the change in mass and temperature. In this chapter, principle of TGA and the instrumentation along with its comprehensive working have been described. The calibration procedure is also given in this chapter. The factors affecting the results along with advantages, disadvantages, and applications have also been described in this chapter.

Keywords

Principle of TGA · Working of TGA · Types of TGA

19.1 Introduction

A technique in which the mass of a substance is measured as a function of temperature, while the substance is subjected to a controlled temperature program is described. This technique is studied under thermal analysis and is used for the estimation of those materials which undergo the mass change when subjected to the thermal events such as decomposition, reduction, and oxidation. It is very important to consider the factors which affect the change in the mass of a sample. The thermobalance is used in TGA. Results are obtained by plotting a graph between the mass change and the temperature. The instrument which is mostly used for TGA is known as "thermobalance." Data obtained from TGA is recorded in the form of curve which is known as "thermogram." The furnace is used to raise the temperature as high as 1000 °C and it is constructed of quartz.

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19.2 Principle

TGA determines the amount and the rate of weight change of a substance with respect to temperature or time in controlled conditions. TGA is a technique in which a change in the weight of a substance is recorded as a function of temperature or time. Change in the temperature affects the sample by changing the mass of the sample. The crystallization, desorption, sublimation, evaporation, oxidation, reduction, and decomposition bring drastic change in the mass of the sample.

19.3 Types of Thermogravimetry

There are three types of TGA, which are as follows:

19.3.1 Dynamic TGA

In this technique, the continuous linear increase in the sample temperature with respect to time is occurred.

19.3.2 Isothermal TGA

In this type of analysis, the temperature of sample is kept constant for a period of time and change in weight is measured.

19.3.3 Quasistatic TGA

In this analysis, the sample is heated until the constant weight is obtained when temperature is increasing in a series.

19.4 Instrumentation

TGA is constructed of the following three important parts and its schematic representation has been illustrated in Fig. 19.1:-

19.4.1 Microbalance

A balance that is designed to measure the very small weights such as parts per millions of a gram.

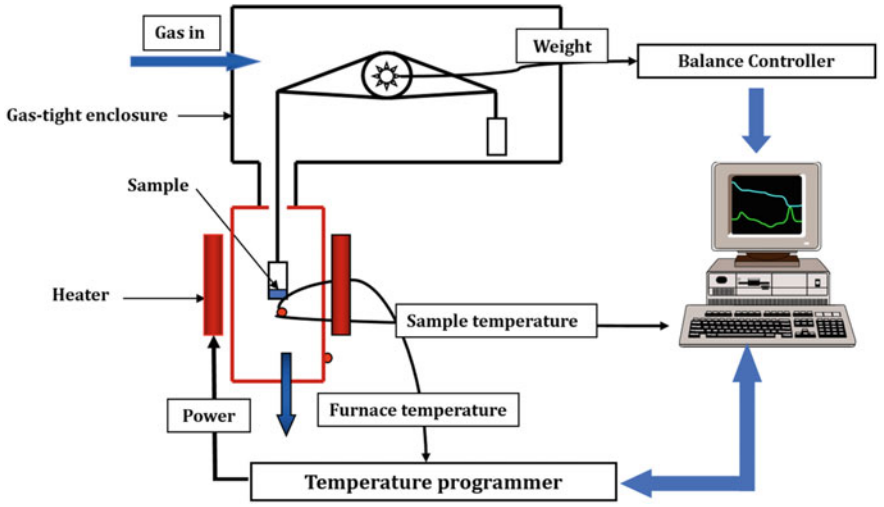

Fig. 19.1 Schematic representation of components of thermo gravimetric analysis

19.4.2 An Auto Sampler

A device that is used for automatic loading of the collected sample into a laboratory instrument. It is usually widely used in spectroscopic and thermal analysis.

19.4.3 Thermocouple

An electronic device that is used for measuring the temperature. It has a pair of wires that is made up of different metals which are joined together and their free ends are attached to a voltmeter that is used to measure the potential difference created between the joining point of the metals.

19.4.4 Furnace

It is an enclosed structure which provides heat to the sample and the sample can be heated at a high temperature.

19.4.5 Temperature Programmer

The temperature of the system is adjusted by entering the pre-planned data. The temperature of the system may increase or decrease step by step.

19.4.6 Recording System

The output obtained from the furnace and microbalance is recorded in the form of chart or using a microcomputer. Nowadays, the recording system is associated with a software, which allows the data to be stored and plot the graph for interpretation of the result efficiently.

19.5 Working of TGA

The sample is loaded on to the microbalance with the help of auto sampler. The thermocouple is placed right above the sample. The temperature of furnace is raised slowly. Then the change in the mass corresponding to the temperature is measured. The thermocouple is used to measure the temperature of sample and change in the weight is measured by finding the beam deflection. During the whole experiment, it is avoided direct contact of sample with thermocouple while the sample is placed in platinum pan. TGA is used to measure the changes in the mass of sample and its scanning is performed in a highly controlled atmosphere. The weight of sample is measured as a function of temperature by thermobalance. The sample is placed in a furnace by hanging with balance and sample is thermally isolated from the furnace. The following points should be considered while working on TGA:

19.5.1 Sample Preparation

For obtaining good data, sample preparation is very important. By increasing the surface area of a sample which is placed in the pan, the resolution and reproducibility of weight loss temperatures can be improved. If the weight of the sample is reduced, then it affects the accuracy of weight measurements. Usually, 10–20 mg of sample is used in most of the applications. If the sample is volatile in nature, then the weight of the sample should be 50–100 mg. It is to be considered that most TGA instruments usually have the baseline drift value of ± 0.025 mg which is equal to $\pm 0.25\%$ of a 10 mg sample substance.

19.5.2 Heating Rate

In most of the cases, the sample is heated at the rate of 20 °C/min. The resolution of overlapping weight loss is improved by decreasing the heating rate. The variable heating rates can also be used in TGA. For this purpose, TGA instrument should be constructed in such a way that it enhances the resolution automatically by decreasing the heating rate during the interval of weight loss.

19.5.3 Purge Gas

Nitrogen is inert in the nature. That's why it can be used to purge samples in TGA because of its nature. Helium is used to provide the baseline. The difference in the oxidative stability of sample components has effect on the result obtained from TGA. Its resolution can be enhanced by using air. When sample components are volatile in nature, then vacuum can be used which improves the separation of components from the start of the decomposition and the volatiles formed from the sample at lower temperature in vacuum.

19.6 Calibration

The instrument is calibrated by using the following steps:

19.6.1 Blank Test

The sample is not placed in pan when the calibration is performed. Only air is passed through the pan and temperature is raised up to 1000 °C at a heating rate of 10 °C/min. The general condition of apparatus should be checked before doing this blank test. When the temperature is increased, then a slight drift in TGA curve is observed. This is responsible for change in convection and buoyancy. Noise can be occurred in TGA curve due to the following reasons. The contact between the sample dish and thermocouple, quartz suspension wire and purge gas feed pipe and weight pan and arid glass cap. The vibration and shock are also responsible for producing noise. A slight decrease in curve is observed in TGA curve when sample pan or suspended wire is contaminated with decomposition product.

19.6.2 Calibration of Mass Changes

TGA is usually used for measuring the weight of the sample by the rate of the weight change of sample. That's why the calibration of absolute weight value of sample is very essential. A 20 mg weight of the sample is calculated with the precision of 10 μg by using a precision balance, and the average value is calculated. The instrument balance control is adjusted to set the automatic zero when furnace is put on and TGA signals are stabilized.

19.6.3 Calibration of Temperature

The temperature of TGA can be calibrated in two ways. One way is the use of the melting point of a pure metal and the second is to use Curie point temperature. In the first procedure, one of the metals is handled in a ribbon shape and then this

metal is placed on TGA suspension wire. The weight of approximately 100 mg is attached with suspended wire tip. By heating, the pure metal is fused and as a result, the weight is reduced. This drop-in weight can be observed in TGA curve. In Curie point temperature technique, the standard substances are verified by International Congress on Thermal Analysis (ICTA). The standard substance which can be used in TGA are ferromagnets and their Curie temperature is different. So, it is necessary to calibrate it by estimating the apparent weight changes at Curie temperatures by permanent magnet. The composition of sample materials and their thermal stability can be predicted from TGA data. It depends upon the mass of the sample changes due to oxidation, dehydration, evaporation, and decomposition. These phenomena may occur when temperature is as high as 1000 °C. The typical example is calcium oxalate hydrate. When it is heated up to 1000 °C, then decomposition takes place which is observed from the curve which shows decomposition occur in three steps. The data of weight loss is measured after half second during the whole experiment.

19.7 Factors Affecting the TG Curve

The factors which may affect the results of TGA are categorized into two major groups which are as follows:

19.7.1 Instrumental Factors

Instrumental factors that affect the TGA curve are as follows:

19.7.1.1 Furnace Heating Rate

The temperature at which the substance (or sample) degrades depends upon the heating rate. When the heating rate is so high, then the decomposition of the substance is also at a high rate. For reproducible and reliable TGA, heating rate of 3.5 °C/min is mostly used. If the heat is endothermic and exothermic, it will be responsible for the sample temperature and increase or decrease in the furnace temperature.

19.7.1.2 Furnace Atmosphere

The temperature at which the sample is decomposed is affected by the atmosphere inside the furnace surrounding the sample. A pure nitrogen gas is passed from cylinder to the furnace for creating highly inert atmosphere.

19.7.2 Sample Characteristics

The characteristics of the sample which affect the experimental procedure are as follows:

19.7.2.1 Weight of Sample

In this technique, usually small weight of the sample is used. Due to the small amount of the sample, temperature gradient will reduce through the sample. If large amount of the sample is used, then deviation from the linearity occurs especially in exothermic reaction.

19.7.3 Particle Size of Sample

The small and uniform shaped particles are usually used in this technique. If large particle or crystal is used, then it may cause rapid weight loss during heating.

19.8 Advantages

1. Any type of solid can be analyzed with the minimal sample preparation.
2. It has high accuracy of balance, high precision of temperature controlling system and atmospheric conditions.
3. It is a convenient and time-saving technique because it has no too much construction requirements.
4. The sample and sample holder can be easily changed.
5. Fast heating rate with good resolution can be maintained.

19.9 Disadvantages

1. Limitation for sample volume.
2. Interpretation of the result is very difficult.
3. It is very sensitive to any change.
4. It cannot optimize the sensitivity and resolution in a single experiment.
5. The quantitative analysis of the individual process is impossible.
6. During the experiment, decomposition of pharmaceuticals occurs. This renders the products which are practically insoluble and mostly sticky in nature and stick inside the wall of a DSC cell.
7. Due to these products, the life of DSC cell is reduced.

19.10 Applications

There is a wide range of applications of TGA which are as follows:

1. It is used in the composition of multi-component system.
2. It can be used for thermal stability of polymers.
3. The oxidative stability studies of polymers can be done by this method.

4. The shelf-life of the finished good product can also be estimated by this technique.
5. The decomposition kinetics of many polymers can also be studied by this method.
6. The effect of corrosive and reactive atmosphere on polymers can be studied.
7. It can be used for determination of moisture and volatile contents in the sample materials.
8. It can also be used for determination of the bound and unbound water in the suspension of milk of magnesia (MoM) which is used as a laxative.
9. The comparison of the generic and a brand MoM can also be studied by this method.
10. In thermal analysis method, it is always preferable to perform a TGA experiment on unknown given sample before performing a DSC experiment (especially for pharmaceuticals).
11. The change in the state of catalyst is determined by using this technique.

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