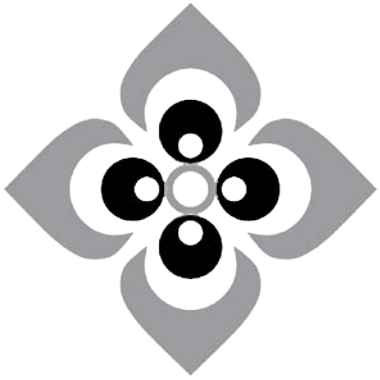


Consortium for Educational Communication

Module on

FLUORESCENCE SPECTROSCOPY & POLARIMETRY

By

NASEER AHMAD BHAT

Department of Food Science & Technology.

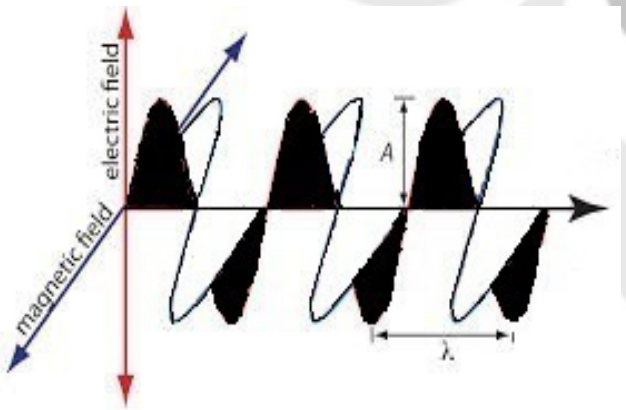
University of Kashmir, Srinagar

Text

SPECTROSCOPY

Introduction

Light is supposed to have dual characteristics i.e., corpuscular and waveform. Thus, a beam of light may be understood as an electromagnetic wave-form disturbance or photon of energy propagated at 3.0×10^8 m/sec, i.e., at the speed of light. The term electromagnet is a precise description of the radiation in that the radiation is made up of an electrical and a magnetic wave which are in phase and perpendicular to each other and to the direction of propagation of wave (Figure 1.1A). The magnitude of electrical vector is denoted by the symbol E and that of magnetic vector is denoted by the symbol H. Figure 1. 1A denotes one ray of plane polarized light. A beam of light from a bulb consists of many randomly oriented plane polarized components being propagated in the same direction. The distance along the direction of propagation for one complete cycle is known as wavelength, 'λ' (Fig. 1.1A). Wave length may be measured in centimeters (cm), micrometers (μm), nanometers (nm), or angstrom units (Å), where 1 nm = 10^{-3} μm = $^{6-}10$ mm = 10^{-7} m, and 1 Å = 10^{-8} cm.



Direction of Propagation

Electric Component

Magnetic component

Figure 1.1 (A) The electromagnetic wave. The magnetic vector (unshaded) and electric vector (shaded) are perpendicular to each other and to the direction of propagation. Wavelength (λ) is the distance

between two crests or troughs.

Sometimes the term frequency 'v' is used rather than wave length to describe a particular radiation. To understand this term it will be helpful to see Figure 1. 1B denoting variation in the electric and magnet field with respect to time at any given location in a plane polarized ray. The number of waves passing through a fixed point on the time axis per second I s known as the frequency, v, of the radiation (usually expressed in Hertz or cycles per second). Frequency shares an inverse relationship with the wave length so that

Where 'c' is the velocity of light, 3×10^8 m/sec

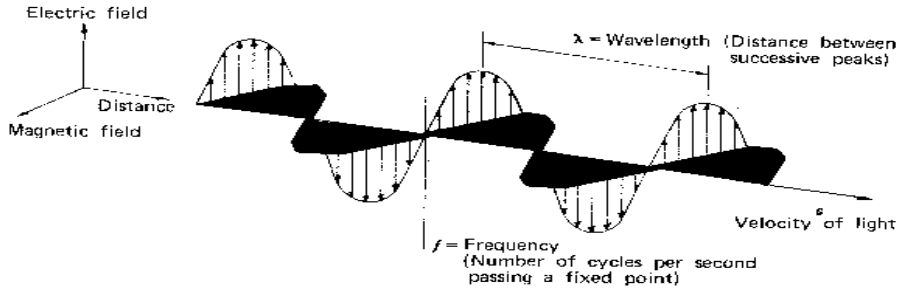


Figure 1.1 (B) Time dependence of electric (shaded) and magnetic unshaded) vectors at a perpendicular location in a plane polarized electromagnetic wave.

Sometimes radiation, mostly in the infra red region, is characterized by another term known as the wave number and denoted by the symbol '□'. Wave number means the number of complete cycles occurring per centimeter.

The energy 'E' of a photon can be related to its wave length and frequency with the help of plank's constant, 'h'

Where c is the velocity of light in vacuum. Remember that wave length and frequency share an inverse relationship; this means that as the wave length increases, the energy of the radiation decreases, while the energy increases with the increase in frequency.

A beam of radiation from an electric bulb consists of several wave lengths and is known as polychromatic. A beam in which all the rays have

the same wave length is known as monochromatic.

Electromagnetic radiation is produced by events at the molecular, atomic, or nuclear level. Oscillations of nuclei and electrons in electrical or magnetic fields, molecular bending and vibration, excitation of orbital electrons, ejection of an inner orbital electron and rearrangement of the other electrons, and nuclear break-up are some of the events which give rise to electromagnetic radiation. Since each of these events differ in terms of the energy involved, the radiation they emit will have different wave lengths. Thus, a complete spectrum of electromagnetic radiation will be produced. A branch dealing with the study of different spectra produced by electromagnetic radiations is referred to as spectroscopy.

Experimental methods of spectroscopy began in the more accessible visible region of the electromagnetic spectrum where the eye could be used as the detector. In 1665, Newton had started his famous experiments on the dispersion of white light into a range of colors using a triangular glass prism. However, it was not until about 1860 when Bunsen and Kirchhoff began to develop the prism spectroscope as an integrated unit for use as an analytical instrument. Early applications were the observation of the emission spectra of various samples in the flame. The visible spectrum of atomic hydrogen had been observed both in the solar spectrum and in an electrical discharge in molecular hydrogen many years earlier, but it was not until 1885 when Balmer fitted the resulting series of lines to a mathematical formula. In this way, there began a close relationship between experimental and theoretical fields of spectroscopy, the experiments providing the results and the relevant theory attempting to explain them and to extrapolate the data. However, theory ran increasingly into trouble as it was based on classical Newtonian mechanics until 1926 onwards when Schrodinger developed quantum mechanics. Even after this breakthrough, the importance of which cannot be overstressed as that theory tended to limp along behind the experiment. Data from spectroscopic experiments, except for those on the simplest atoms and molecules, were easily able to outstrip the predictions of theory, which was almost always limited by the approximations that had to be made in order that the calculations are manageable. It was only from about 1960 onwards that the situation changed as a result of the availability of large, fast computers requiring many fewer approximations to be made. Nowadays it is not uncommon for predictions to be made of spectroscopic and structural properties of fairly small molecules that are comparable in accuracy to those obtainable from experiment.

Spectroscopy is a general term that deals with the interactions of various types of radiation with matter. Historically, the interactions of interest were between electromagnetic radiation and matter, but now spectroscopy has

been broadened to include interactions between matter and other forms of energy. Examples include acoustic waves and beams of particles such as ions and electrons. Spectrometry and spectrometric methods refer to the measurement of the intensity of radiation with a photoelectric transducer or other type of electronic device. The most widely used spectrometric methods are based on electromagnetic radiation, which is a type of energy that takes several forms, the most readily recognizable being light and radiant heat. The other forms include gamma rays and X-rays as well as ultraviolet, microwave, and radio-frequency radiation.

DEFINITION

Spectroscopy is the analysis of the electromagnetic radiation emitted, absorbed or scattered by atoms or molecules as they undergo transitions between two energy levels. The frequency, *v,* of the electromagnetic radiation associated with a transition between a pair of energy levels *E*1 and *E*2 is given by:

$$h\nu = |E1 - E2| = |\Delta E|$$

Types of Spectroscopy

Spectroscopy is broadly classified into three main types depending on:

1. Type of radiative energy.
2. Nature of the interaction.
3. Type of material.

1. **Type of radiative energy:** Types of spectroscopy are distinguished by the type of radiative energy involved in the interaction. In many applications, the spectrum is determined by measuring changes in the intensity or frequency of this energy. The types of radiative energy studied include:

- **Electromagnetic radiation** was the first source of energy used for spectroscopic studies. Techniques that employ electromagnetic radiation are typically classified by the wavelength region of the spectrum and include microwave, infrared, near infrared, visible and ultraviolet, x-ray and gamma spectroscopy.
- **Particles**, due to their de Broglie wavelength, can also be a source of radiative energy and both electrons and neutrons are commonly

used. For a particle, its kinetic energy determines its wavelength.

- **Acoustic spectroscopy** involves radiated pressure waves.
- **Mechanical methods** can be employed to impart radiating energy, similar to acoustic waves, to solid materials.

2. **Nature of the interaction:** Types of spectroscopy can also be distinguished by the nature of the interaction between the energy and the material. These interactions include:

- **Absorption** occurs when energy from the radiative source is absorbed by the material. Absorption is often determined by measuring the fraction of energy transmitted through the material; absorption will decrease the transmitted portion.
- **Emission** indicates that radiative energy is released by the material. A material's blackbody spectrum is a spontaneous emission spectrum determined by its temperature. Emission can also be induced by other sources of energy such as flames or sparks or electromagnetic radiation in the case of fluorescence.
- **Elastic scattering** and **reflection** spectroscopy determine how incident radiation is reflected or scattered by a material. Crystallography employs the scattering of high energy radiation, such as x-rays and electrons, to examine the arrangement of atoms in proteins and solid crystals.
- **Impedance spectroscopy** studies the ability of a medium to impede or slow the transmittance of energy. For optical applications, this is characterized by the index of refraction.
- **Inelastic scattering** phenomena involve an exchange of energy between the radiation and the matter that shifts the wavelength of the scattered radiation. These include Raman and Compton scattering.
- **Coherent** or resonance spectroscopy is a technique where the radiative energy couples two quantum states of the material in a coherent interaction that is sustained by the radiating field. The coherence can be disrupted by other interactions, such as particle collisions and energy transfer, and so often require high intensity radiation to be sustained. Nuclear magnetic resonance (NMR) spectroscopy is a widely used resonance method and ultrafast laser methods are also now possible in the infrared and visible spectral regions.

3. **Type of material:** Spectroscopic studies are designed so that the radiant energy interacts with specific types of matter.

Atoms

Atomic spectroscopy was the first application of spectroscopy developed. Atomic absorption spectroscopy (AAS) and atomic emission spectroscopy (AES) involve visible and ultraviolet light. These absorptions and emissions, often referred to as atomic spectral lines, are due to electronic transitions of outer shell electrons as they rise and fall from one electron orbit to another. Atoms also have distinct x-ray spectra that are attributable to the excitation of inner shell electrons to excited states.

Atoms of different elements have distinct spectra and therefore atomic spectroscopy allows for the identification and quantitation of a sample's elemental composition. Robert Bunsen and Gustav Kirchhoff discovered new elements by observing their emission spectra. Atomic absorption lines are observed in the solar spectrum and referred to as Fraunhofer lines after their discoverer. A comprehensive explanation of the hydrogen spectrum was an early success of quantum mechanics and explained the Lamb shift observed in the hydrogen spectrum led to the development of quantum electrodynamics**.**

Modern implementations of atomic spectroscopy for studying visible and ultraviolet transitions include flame emission spectroscopy, inductively coupled plasma atomic emission spectroscopy, glow discharge spectroscopy, microwave induced plasma spectroscopy, and spark or arc emission spectroscopy. Techniques for studying x-ray spectra include X-ray spectroscopy and X-ray fluorescence (XRF).

Molecules

The combination of atoms into molecules leads to the creation of unique types of energetic states and therefore unique spectra of the transitions between these states. Molecular spectra can be obtained due to electron spin states (electron paramagnetic resonance), molecular rotations, molecular vibration and electronic states. Rotations are collective motions of the atomic nuclei and typically lead to spectra in the microwave and millimeter-wave spectral regions; rotational spectroscopy and microwave spectroscopy are synonymous. Vibrations are relative motions of the atomic nuclei and are studied by both infrared and Raman spectroscopy. Electronic excitations are studied using visible and ultraviolet spectroscopy as well as fluorescence spectroscopy.

Crystals and extended materials

The combination of atoms or molecules into crystals or other extended forms leads to the creation of additional energetic states. These states are numerous and therefore have a high density of states. This high density often makes the spectra weaker and less distinct, i.e., broader. For instance,

blackbody radiation is due to the thermal motion of atoms and molecules within a material. Acoustic and mechanical responses are due to collective motion as well.

Although, pure crystals can have distinct spectral transitions and the crystal arrangement, these also have an effect on the observed molecular spectra. The regular lattice structure of crystals also scatters x-rays, electrons or neutrons allowing for crystallographic studies.

Nuclei

Nuclei also have distinct energy states that are widely separated and lead to gamma ray spectra. Distinct nuclear spin states can have their energy separated by a magnetic field, and this allows for NMR spectroscopy.

Fluorescence spectroscopy or Spectrofluorimetry

Introduction

The phenomenon of luminescence was first studied by Stoke's in about 1852. Stoke's noticed that when fluorspar was placed in the sun it seemed to glow. The orange jackets of highway workers that appear very bright even on cloudy days, road signs that "glow in the night," and an afterglow when a television set is turned off, are modern examples of this phenomenon. Luminescence is a general term to describe systems that can be made to glow. Such systems can be classified according to the glow-producing mechanism. The two major divisions of present-day analytical importance are fluorescence and phosphorescence.

In general, compounds that fluoresce or phosphoresce contain either an electron-donating group (amines, alcohols, and hetero atoms) or multiple conjugated double bonds (aromatic rings). Notice that these groups contain either nonbonding or л electrons. The presence of groups that tend to withdraw electrons (e.g., carboxyl, azo, the halides, and nitro groups) usually destroy fluorescence.

Fluorescence spectroscopy is becoming more and more popular instrumental technique for providing direct and indirect exploratory information about chemical and physical properties of food products. The primary reasons are the high specificity, the high sensitivity, and that several substances inherent to food systems exhibit intrinsic fluorescence, such as proteins, vitamins, secondary metabolites, pigments, toxins, and flavoring compounds. Fluorescence spectroscopy analysis often involves the use of an extrinsic probe developed for specific analyses or requires a sample preparation step before the fluorescence measurement to reduce the effect of other influencing parameters. However, for food analysis, the intrinsic fluorescence of the intact food, called autofluorescence, can be measured. The use of autofluorescence increases the speed of analysis considerably, facilitates nondestructive analysis, and makes fluorescence a potential method for online or at-line applications. Furthermore, direct measurements on foods enhance the scientific exploitation of the measurements, allowing exploratory studies of the more complex relationships within the sample.

BASIC PRINCIPLE

The phenomenon whereby a molecule, after absorbing radiations, emits radiation of a longer wavelength is known as fluorescence. Thus a compound which absorbs in the ultraviolet range might emit radiation in the visible range. This shift towards a longer wavelength is known as Stoke's shift.

S_2

Excitation

S_1

Emision

Rayleigh

S_v

Raman scatter

S_0

FIGURE 2. 1(A) Jablonski diagram: S_0 denotes the electronic ground level; S_1 and S_2 are electronically excited singlet states and S_v is a virtual electronic state. The horizontal lines indicate the vibrational levels within each electronic state and the vertical arrows illustrate the various forms of electronic transitions and emission of light.

When an atom or molecule absorbs light, the energy of photon absorbed lifts an electron to a higher orbital. The excited electron can now return to the ground state in either of the two ways. It might do so in one single step in which case it will emit light of the same wavelength that is absorbed. The electron might, however, return to the ground state in a step-wise manner through intermediate energy levels, emitting quanta of radiation corresponding to each energy step (Fig. 2.1A). Since each quantum will have a smaller amount of energy, the radiation emitted will have a longer wavelength than the original exciting radiation. It is obvious that the emitted light will have many different wavelengths corresponding to each intermediate energy level the electron adopts on its journey to the ground state. Therefore, fluorescence spectra are band spectra. They are usually independent of the wavelength of the radiation absorbed. Each molecule or substructure able to emit fluorescence, called a fluorophore, can only be excited by light of specific wavelengths and will only emit light at characteristic higher wavelengths when the molecule returns to its more stable ground state. Thus, all fluorescent molecules have unique fluorescence properties. In the ideal case, excitation and emission spectra are identical and can be considered as mirror images of each other due to the same electronic transitions taking place. Moreover, the visual appearance of the emission spectrum does not depend on the excitation wavelength, i.e., if the excitation light is at a wavelength different from the absorption maximum, the molecule will absorb less radiation and the emission will

be of lower intensity. This process will result in broad emission/excitation peaks when measuring emission spectra for a number of neighboring excitation wavelengths. The use of both excitation and emission spectra gives fluorescence spectroscopy a high degree of selectivity. Two molecules with similar excitation spectra may have different emission properties.

Fluorescence is sometimes called luminescence, which covers the emission of light from molecules in an electronically excited state. Phosphorescence is a similar, but slower luminescence phenomenon. The excited molecule goes through an intermediate excited triplet state from where light is emitted by returning to the ground state. Wavelength (nm) The instrumentation and spectral region for measurement of phosphorescence are almost identical to fluorescence spectroscopy.

FACTORS AFFECTING FLUORESCENCE

The fluorescence properties of a molecule depend strongly on the sample matrix. The concentration, turbidity, local molecular environment, pH, and temperature influence the measurement due to phenomena such as quenching, inner-filter effects, scatter, etc. Some of these will be explained in the following subsections.

1. **QUENCHING**

   The fluorescence intensity may decrease as an effect of intra- or intermolecular interactions either within the fluorescent molecule itself, in the solvent, or with other molecules in the sample matrix. This phenomenon is called fluorescence quenching. Quenching can be either static or dynamic. Static quenching takes place when the fluorescent molecule forms a nonfluorescent complex with the quencher molecule, which inhibits the formation of an excited state. While static quenching does not depend on diffusion or molecular collisions, dynamic quenching depends on such molecular movements. It is caused by deactivation of the excited state by contact with another molecule in the sample matrix called the quencher. Oxygen, heavy metals, halide ions, organic and inorganic nitro compounds as well as intramolecular interactions are known to induce dynamic quenching. The molecule returns to the ground state without fluorescence emission and without chemical modification. Higher temperatures may also lead to dynamic quenching, as the increased molecular velocities give rise to more molecular collisions.

2. **INNER FILTER**

   Another type of mechanism that will reduce the measured fluo-

rescence intensity is the so-called inner-filter effects, which are due to absorption of emitted light by either the fluorophore itself or by chromophores in the sample matrix. Inner filter occurs when a chromophore reabsorbs the emitted fluorescence or when a nonfluorescent chromophore absorbs parts of the excitation light. The result is decreased fluorescence intensity or distortion of band shapes. Concentration quenching and inner-filter effects can normally be handled by diluting the sample or by reducing the optical path length. However, diluting the sample reduces the concentration of other relevant fluorophores and changes the molecular interactions of the intact food matrix.

3. CONCENTRATION

Like absorption spectroscopy, fluorescence spectroscopy depends linearly on the concentration, but it has to be within a certain concentration range in order to avoid artifacts such as quenching and inner-filter effects. For transparent solutions with absorbance below 0.05, the fluorescence intensity is linearly related to the concentration of the fluorophore. In this case, the fluorescence intensity, $\mathbf{I_f}$, depends on the intensity of the incident light, $\mathbf{I_0}$, the molar absorptivity, ϵ, the fluorescence quantum yield, □, the optical length, l, and the molar concentration of the fluorophore, c:

The quantum yield is defined as the number of emitted photons relative to the number of absorbed photons. Together with the molar absorptivity, it is a measure of the effectiveness of the fluorophore.

4. MOLECULAR ENVIRONMENT

In addition to concentration, fluorescence intensity depends on the molecular environments such as polarity, pH, temperature, etc. For example, tryptophan residues buried in the interior of a protein exhibit different fluorescence than tryptophan exposed to the hydrophilic solvent on the surface. Figure 3.1 shows an example of emission spectra measured on the milk proteins: α-lactalbumin,

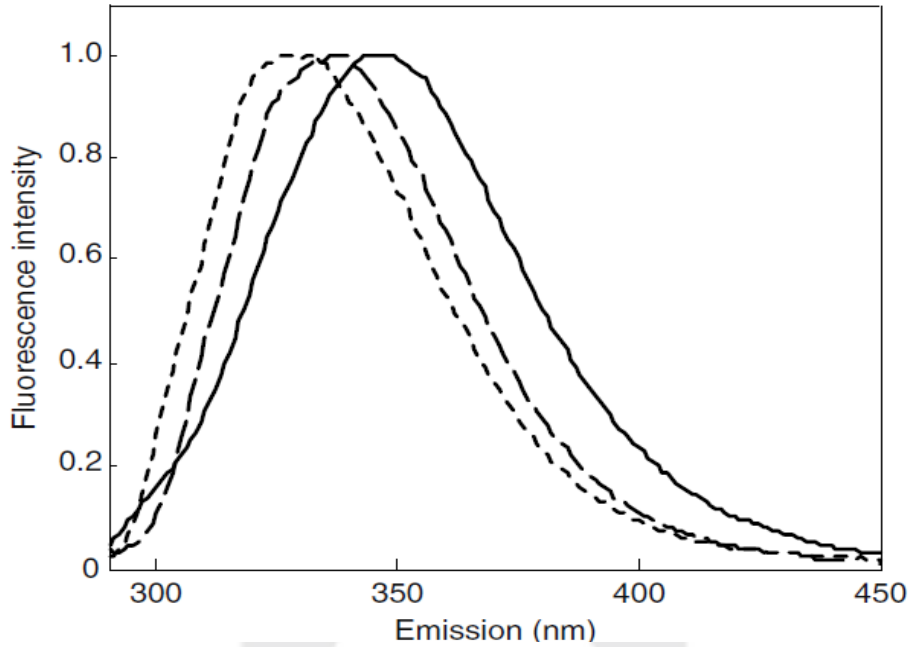


FIGURE 3.1 Fluorescence emission spectra of a-lactalbumin (dotted), b-lactoglobulin (dashed), and casein (solid) dissolved in water. Excitation was set to 282 nm.

β-lactoglobulin and casein. Casein, which is the least structured protein, has emission maximum at the highest wavelength. In casein, tryptophan is more exposed to the solvent, which in this case is water. A similar change in emission spectra is observed when comparing a fluorophore dissolved in a hydrophilic solvent, such as water, and the same fluorophore dissolved in a hydrophobic solvent, i.e., an organic solvent. An increase in hydrophobicity of the fluorophore's environment entails relaxation of the excited state to a lower vibrational level before emitting fluorescence. This results in emission of lower energy than obtained from more polar solvents. These differences make it possible to study conformational protein changes as well as denaturation and interaction of proteins with other food components.

5. SCATTER

Scatter of light is a phenomenon that may disturb the measured fluorescence signal. Light is primarily scattered by small particles in the samples and is observed by the fluorescence detector as false emitted light. Light scatter disturbs the fluorescence measurements when the Stoke's shift is small and the signal overlaps with the analyte emission. There are two main scatter phenomena: elastic Rayleigh scatter and inelastic Raman scatter. Elastic Rayleigh scatter occurs at the same wavelength as the excitation wavelength (Figure 3.2). In Rayleigh scatter there is no electronic interaction between the light and matter, for which reason the Rayleigh scatter will be of the same energy and found at the same

wavelengths. The intensity of the Rayleigh scatter varies with the fourth power of the wavelength. Thus, the effect can be minimized using higher excitation wavelengths. Second-order Rayleigh scatter appears at twice the excitation wavelength.

Inelastic Raman scatter is much weaker than Rayleigh scatter and is often not observed if the fluorescence intensity is high. Raman scatter appears at longer wavelengths than the excitation wavelength with a constant distance in frequency (not wavelength) to the Rayleigh scatter peak. It is caused by vibrational interactions with the solvent. Raman scatter is important because it may overlap with the fluorescence signal and especially at low fluorescence intensities, it can make up a large part of the measured signal. Correction of Raman scatter can be performed by subtracting the fluorescence signal of the pure solvent from the measured sample signal.

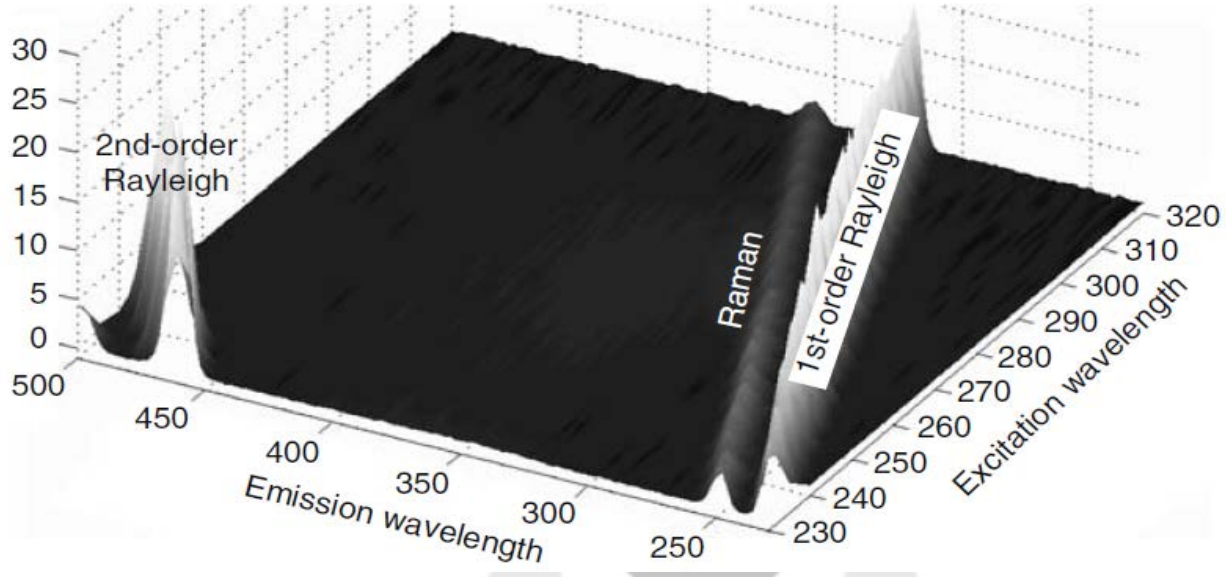


FIGURE 3.2 Appearance of Rayleigh and Raman scatter in a fluorescence landscape.

FLUOROMETRY: THEORY AND INSTRUMENTATION

Fluorometry is an important analytical tool for the determination of extremely small concentrations of substances which exhibit fluorescence. Beer-lambert law which states that the amount of light absorbed (absorption or extinction) is proportional to the concentration of the absorbing substance and to the thickness of the absorbing material (path-length), is also applied to fluorometry in the following form

Where ϵ_f is the absorptivity of the fluorescent material. C is the concentration of the substance and b is the path length. The values of intensities of the incident radiant energy and the transmitted energy are indicated by $\mathbf{I}_{solvent}$ and $\mathbf{I}_{sample}$ respectively. The intensity of radiation absorbed is given by $\mathbf{I}_{solvent} - \mathbf{I}_{sample}$. The intensity of fluorescence is given by:

)

Where **k** is the proportionality constant (<1). Thus, if $\mathbf{kI}_{solvent}$ is written as **Fn** the relationship becomes

$F = Fn\ (1-10^{-\epsilon_f\ \mathbf{Cb}})$

Rearranging

Therefore,

For $\epsilon_f\mathbf{Cb} \leq 0.01$, $F \approx 2.303\ \mathbf{kI}_{solvent}\epsilon_f\mathbf{Cb}$. This equation holds good for concentrations as a few p.p.m.

The instrumentation of a spectrofluorometer differs from that of the spectrophotometer in two important respects besides other minor variations.

a) There are two monochromators instead of one as in a spectrophotometer; one monochromator is placed before the sample holder and one after it, and

b) As fluorescence is maximum between 25-30°C, the sample holder has a device to maintain the temperature.

The main components of a spectrofluorometer, indicated in Figure 4.1 are:

I. a continuous source of radiant energy (mercury lamp or xenon arc);

II. a monochromator usually a prism, to choose the wavelength with which the sample is to be irradiated;

III. a second monochromator, placed after the sample, enables the determination of the fluorescent spectrum of the sample;

IV. a detector, usually a photomultiplier suited for wavelengths greater than 500 nm; and

V. an amplifier.

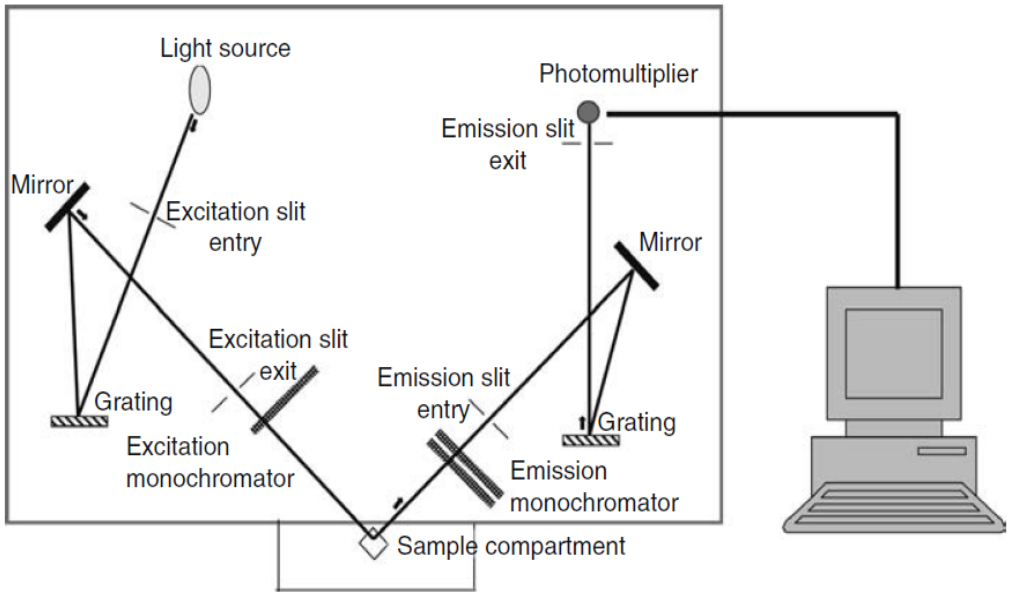


Figure 4.1 Schematic overview of a fluorescence spectrophotometer

The fluorescent radiation emitted by the sample is given off in all directions, but in most instruments the sample is viewed at right angles to the incident beam.

APPLICATIONS

The more common applications of spectrofluorimetry include qualitative analysis (comparison of fluorescence spectra and absorption spectra gives a fair idea about the identity of a compound); quantitative analysis (applications include assay of riboflavin, thiamine, hormones such as cortisol, estrogen, serotonin and dopamine, organophophorus pesticides, tobacco smoke carcinogens, drugs such as lysergic acid and barbiturates, porphyrins, cholesterol, and even some metal ions); and studies on protein structure (FAD containing proteins). However, more important applications of spectrofluorometery are given below.

I. **Intracellular free calcium concentration assay:** Quin-2 (an EGTA

derivative), Quin-2 AM, and Fura-2 are three fluorescent probes which allow us to assay intracellular free calcium concentration. These probes permeable to the plasma membrane and upon entering the cytosol combine with calcium (chelation). This chelation gives rise to fluorescence whose magnitude is proportional to the free Ca^{2+} concentration in the cytosol. These studies assume importance because of the role of calcium in controlling cellular metabolism.

II. **Fluorescent probes and studies on membrane structure:** Fluorescent anilionaphthalien-8-sulphonate (ANS) and N-methyl-2-anilino.6-naphthalene sulphonate (MNS) contain both charged and hydrophobic areas and therefore locate at the water lipid interface of the membrane. Such compounds provide important information about this interface. Studies with ANS have shown that structural changes occur in mitochondrial membrane during oxidative phosphorylation. The probes have also yielded much information about the structural features of the plasma membrane.

III. **Assay of membrane potential:** Membrane potential of excitable cells is regulated within strict limits. Changes in this potential regulate ion entry into the cells. These membrane potential changes can be monitored by using fluorescent probes such as Di-S-C_3-(5), and merocyanine 540 (the latter is not so satisfactory).

IV. **Fluorescent microscopy:** Spectrofluorimetry when combined with a microscope allows the determination of subcellular location of fluorescent compounds or of materials which bind fluorescent dyes. This technique has given important information in the field of immunology and pharmacology. Thus, presence of pathological immune complexes may be detected with the help of FITC (fluorescein isothiocyanate) conjugates. The presence of an antigen on the cell surface may be detected by a fluorescent labelled antibody.

V. Since fluorescent emission is extremely sensitive to local environment, it can be used to monitor the kinetics and thermodynamics of the incorporation of a particular subunit or substrate into macromolecular assembly. It is being used to obtain definitive information about the distance between pairs of loci in a macromolecular assembly.

Apart from these applications fluorescence has been applied on various kinds of food from fish and meat to fruit and vegetables. However, it is almost the same fluorophores that make up the fluorescence emission in the different food items. Protein fluorescence is one of the most frequently studied fluorophores in food due to the strong emission properties of tryptophan. Tryptophan fluorescence has been used as an indicator of protein structure in dairy products, evaluated by minor shift in the emission

maximum. Furthermore, it has been correlated to the texture of meat emulsions and sausages and to meat. Many other food products exhibit tryptophan fluorescence, including wheat, beer, and sugar.

In the following subsections, four specific examples of food fluorescence applications are described in more detail.

a) Direct Fluorescence Measurements

Relatively few studies of food product have shown the direct measurement of the fluorophore concentration from fluorescence spectroscopic data. Riboflavin is a highly fluorescent molecule with excitation maxima at 270, 370, and 450 nm and emission maximum in the range 525–531 nm. The content was determined chemically in plain yoghurt exposed to light for up to 35 days and subsequently predicted from fluorescence measurements by chemometrics. Squared correlation coefficients between measured and predicted riboflavin were 0.97 and the error was estimated to 7% of the mean riboflavin content in the samples (Fig. 5.1).

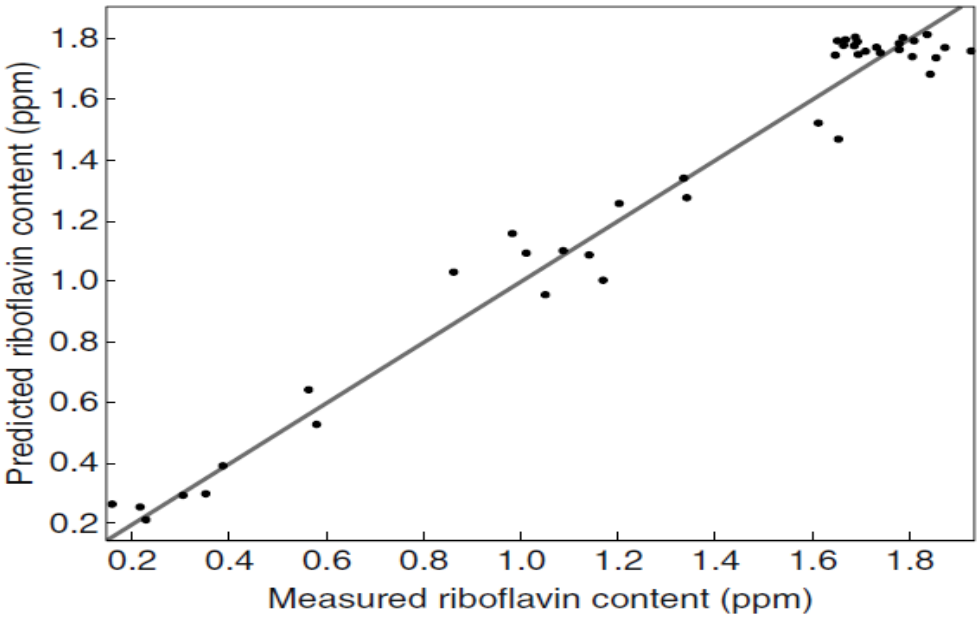


Figure 5.1 Predicted riboflavin content versus the chemically measured values. The predictions were obtained by PLS calibration using scores obtained from a PARAFAC model as X-variables.

b) **Indirect Fluorescence Measurements**

Lipid oxidation is an important area within food research, since exposure of products such as cheese, meat, and other lipid-containing products to light and oxygen for a long period leads to off flavor formation, discoloration, nutrient loss, and formation of toxic compounds, which rapidly impair product quality. Several methods are applied for measuring lipid oxidation in foods, including peroxide value, formation of thiobarbituric reactive substances, iodine value, volatile compounds by gas chromatography (GC), and radicals by electron spin resonance (ESR). However, these determinations are all rather time consuming and strongly invasive. Fluorescence measurements have shown potential to predict some of these oxidation parameters. For example, fluorescence measurements of frying oil during production of spring rolls were correlated to the deterioration. In this industrial study, one frying oil batch was used for 20 days after which the plant was cleaned and restarted with fresh plant oil. Daily oil samples collected during the 4 weeks of use were analyzed with traditional chemical oxidation measurements and fluorescence spectroscopy. Fluorescence landscapes of a fresh frying oil and frying oil that was discarded showed that the two-component system with emission maxima at 475 and 660 nm degenerated to a one-component system with an emission maximum at approximately 585 nm (Figure 5.2). Five emission spectra with excitation set to 395, 420, 440, 500, and 530 nm were measured and used for predicting chemically measured lipid oxidation parameters such as triglycerides, anisidine value, iodine value, and free fatty acids. These spectral settings were chosen based on the excitation and emission maxima of the fluorescence landscapes (Figure5.2). Squared correlation coefficients between reference measurements and predicted values varied between 0.91 and 0.97 and the errors varied from 2% to 30%. The best prediction was obtained for triglyceride, but even for prediction of anisidine value, the fluorescence measurements gave much better results than those obtained using vibrational spectroscopy.

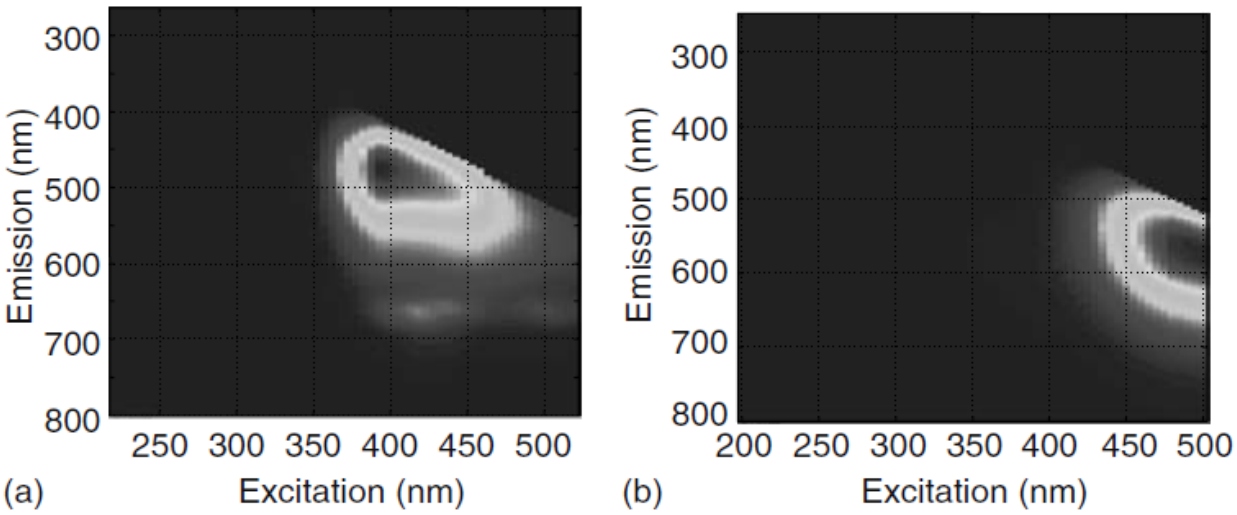


Figure 5.2 Fluorescence landscapes of frying oils: (a) fresh oil and (b) the oil that was discarded after 4 weeks of use.

c) **Food authenticity** is another issue that is becoming increasingly important because the food purchased must match the description. For instance, the content should be equal to what is stated in the declaration, and the geographical, vegetable or animal origin should be as described. The high sensitivity of fluorescence spectroscopy makes it possible to detect compounds in ppb levels. It has been reported that even concentrations down to ppt have been measured. Thus, it has shown potential for detecting adulteration, fraud, etc. A few studies have been conducted on food items such as fruit juice, vegetable oil, honey and milk.

d) FLUORESCENCE AND PROCESS ANALYTICAL TECHNOLOGY

Process analytical technology (PAT) is a new concept introduced within the pharmaceutical industry, but the principle focus has been on the food industry for a number of years. Basically, PAT refers to measuring the relevant information at the relevant time during the production. It can be the incoming raw material, some process streams or process parameters or the finished product. The goal of PAT is through massive internal quality control by remote spectroscopic monitoring to ensure final product quality and to improve production efficiency.

One example of a food PAT application is the monitoring of the beet sugar production by fluorescence spectroscopy. The sugar, sucrose, is not itself fluorescent; however, impurities in both crystalline sugar and sugar solutions make up a strong fluorescence emission. This was recognized already in the 1940s where fluorescence occurring upon il-

lumination with ultraviolet light was applied for quality inspection and determination of impurities. More dedicated experiments showed that fluorescence of sugar stems from tyrosine, tryptophan, a polyphenolic compound as well as colorant polymers formed in Maillard reactions during the sugar processing. To analyze the sugar production, a number of emission spectra of sugar dissolved in water were measured every 8 h during the sugar campaign that lasted for about 3 months. Chemometric data analysis revealed the presence of four fluorescent compounds in the samples, which all displayed a weekly periodicity with a tendency of increased concentrations during weekends in the beginning of the campaign (Figure 5.3).

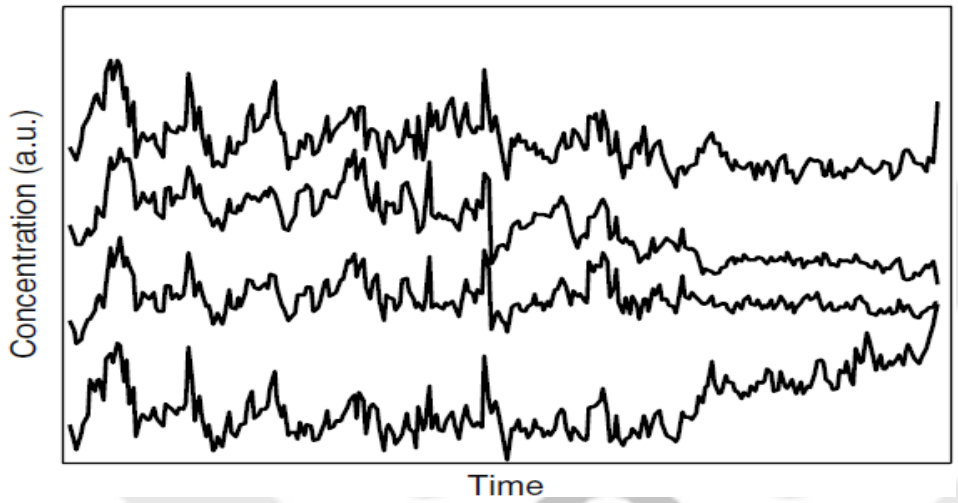


Figure 5.3. Relative concentrations of the four fluorophores in sugar versus the time. For visualization these are separated on the concentration scale.

This increase was suggested to be due to longer storage time in the weekends, resulting in increased temperature and microbiological and enzymatic activity.

POLARIMETRY

Introduction

Polarimetry was discovered by Étienne-Louis Malus, a French engineer who was studying reflective glass. Several years later another Frenchman, Jean-Baptiste Biot, found that molecules such as sugar could rotate polarized light as well. It was not until 1874 when Dutch chemist Jacobus Henricus van't Hoff proposed that carbon's tetrahedral structure was responsible for the optical activity (the ability of many organic compounds to rotate plane-polarized light).

Optical activity is a property unique to chiral substances, for example 2-butanol, which possess a chiral centre (one carbon bound to four different ligands). Figure 6.1 illustrates that 2-butanol exists as two mirror-image isomers, or enantiomers. The atomic connectivity in the S-isomer is identical

to that of its mirror-image R-isomer, except that two of the groups attached to carbon were interchanged.

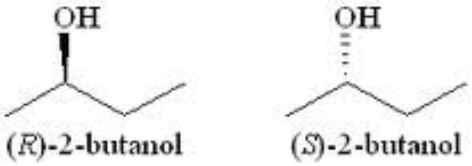


Fig. 6.1 Enantiomers of 2-butanol

The R and S designations are based on the Cahn-Ingold-Prelog rules for assigning priority to substituent groups. Visualize the smallest substituent pointing downward below the plane of the paper or computer screen. The three remaining groups are ranked by molecular weight. If the direction of the heaviest, next heaviest, and lightest group is clockwise the molecule is designated as R; if counter-clockwise the designation is S. When the d and l isomers are present in exactly equal concentrations they are still chiral, but their rotations cancel out, the sample is referred to as a racemate or racemic mixture.

Definition

Polarimetry is a sensitive nondestructive technique for measuring the optical activity exhibited by inorganic and organic compounds. A compound is considered to be optically active if linearly polarized light is rotated clockwise (+) or counterclockwise (-) when passing through it. The amount of optical rotation is determined by the molecular structure and concentration of chiral molecules in the substance.

Let us examine in more detail the light coming from the lamp you are using to read this page. The radiation coming from the lamp has an electric component and a magnetic component, and they act as if they are at right angles to each other. One such ray might be represented as in Fig. 6.2.

OBLIQUE VIEW Electric Component END ON VIEW

Magnetic

Component

Fig. 6.2 Components of radiation.

However there are millions of rays coming from your lamp and the direction of electric and magnetic components are purely random and may look like Fig. 6.3A. This radiation is said to be unpolarized. If, however by some means we can get all the rays to have their electric and magnetic components all in the same direction, then the radiation is plane polarized, as shown in Fig. 6.3B. Compounds that can rotate polarized light are said to optically active.

Fig. 6.3A Unpolarized radiation

Fig. 6.23B Polarized radiation

Unfortunately no correlation exists between the absolute configuration of the molecule (e.g. R- or S-) and the direction in which it rotates polarized light. Molecules that shift the angle clockwise are known as dextrarotatory ("right-turning"), d or (+), while those that shift the angle counter-clockwise are called levorotatory ("left-rotating"), l, or (-). Predicting the precise rotation of a molecule with more than one chiral centre is difficult since both chiral centers contribute to optical rotation.

There are a number of atoms that display optical isomerism, including nitrogen and phosphorus, but the simplest case to consider is that of an sp^3 hybridized carbon atom with four different substituents attached to it (Figure 6.4). A carbon like this is said to be chiral and to display the property of chirality. If the four substituents are different, a pair of non-superimposable mirror image forms can be drawn. These two isomers are called enantiomers. A chiral compound always has an enantiomer, whereas an achiral compound has a mirror image that is the same as the original

molecule.

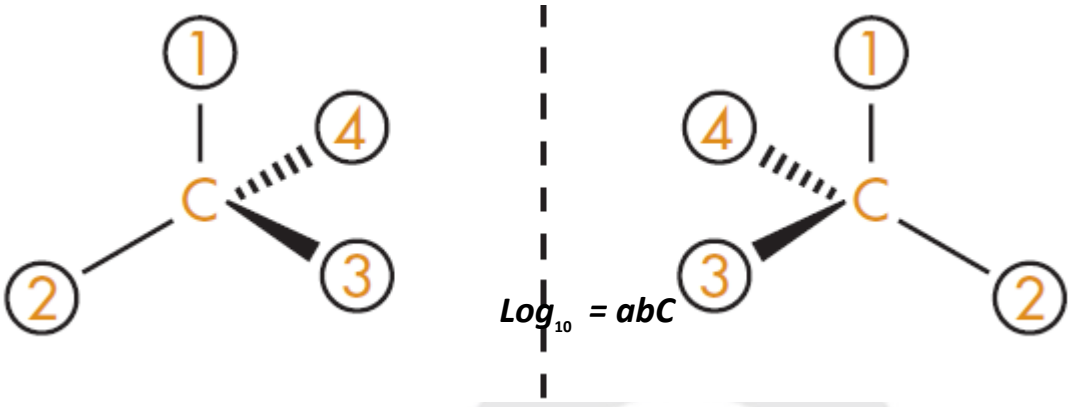


Fig. 6.4 Chiral Carbon atoms

Enantiomers have identical or nearly identical physical properties unless a reagent or technique is used that is itself chiral. For example, the two enantiomers in Figure 6.4 will have the same boiling point, melting point, refractive index and density since these are bulk effects and cannot discriminate between the two enantiomers. Differences between enantiomers only become apparent when they interact with chiral reagents such as the active sites of enzymes or the chiral stationary phase of an HPLC column.

In the laboratory, the technique of polarimetry is used to distinguish between enantiomers and to measure the extent to which each enantiomer rotates the plane of plane-polarised light. The optical instrument used for determining the polarization properties of light beam and samples is called a polarimeter.

Polarimeter

A polarimeter is a device used for the measurement of optical rotation and its scheme is depicted in Fig. 6.5.

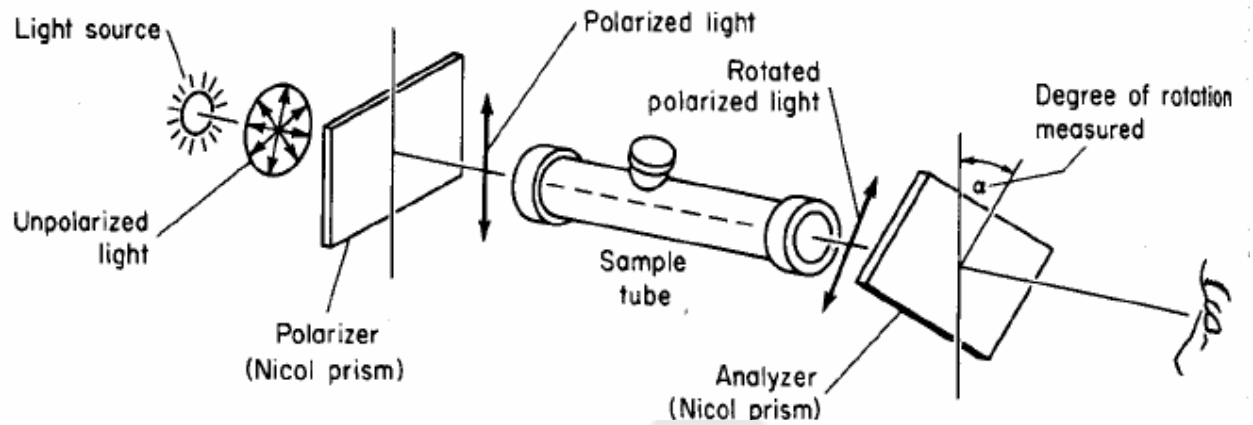


Figure 6.5. Components of a polarimeter

The main components of the polarimeter are a light source (usually a sodium vapour lamp), polarizer (Nicol prism), analyzer (Nicol prism) and the sample tube. The polarizer is used to obtain polarized light. The analyzer is used to measure the angle of rotation. The sample tube should be filled with the sample in a way that after the filling a convex surface of the liquid is formed on the tube thread. This surface is then "cut off" with a lens. This procedure should be performed fast in order to avoid the formation of gas bubbles in the sample tube. After screwing the tube thread the filling of the tube is checked, the tube is wiped and inserted into the polarimeter.

APPLICATIONS

I. The industries that produce or use chiral organic molecules in pure or diluted form, rotations of mixtures, for example of food ingredients, perfumes, flavourings, chemicals, pure or formulated pharmaceuticals are observed. In these situations, polarimetry provides a rapid, reliable, quality check that eliminates the need of using conventional analysis like liquid chromatography which can take an hour to do what the polarimeter accomplishes in minutes.

II. Polarimetry provides an additional check on a pure substance before it is added to an expensive batch to determine the ingredient's concentration or purity. For example a 25% glucose syrup will have an observed rotation that is five-sixths that of a 30% syrup.

III. The optical rotation of a mixed-component ingredient, intermediate, or finished product will have a characteristic optical rotation that may arise from the presence of several chiral compounds. Once a standard

is determined for the composite observed rotation, one can establish quality criteria based on optical rotation. In these situations, the polarimeter measurement becomes a type of screen for further testing to determine which ingredient is out of the specification.

IV. Polarimetry is used by organic chemists to test the effectiveness of catalysts and asymmetric synthetic processes.

V. Many optically active chemicals such as tartaric acid, are stereoisomers, a polarimeter can be used to identify which isomer is present in a sample. If the polarized light is rotated to the left, it is a levo-isomer, and to the right, a dextro-isomer. It can also be used to measure the ratio of enantiomers in solutions.

VI. Concentration and purity measurements are especially important to determine product or ingredient quality in the food & beverage and pharmaceutical industries. Samples that display specific rotations that can be calculated for purity with a polarimeter include:

- Steroids
- Diuretics
- Antibiotics
- Narcotics
- Vitamins
- Analgesics
- Amino Acids
- Essential Oils
- Polymers

VII. Starches are the most abundant substances in nature and used in various sectors of the food and pharmaceutical industry as well as the building sector. Polarimetric quality control of starch therefore is important in various industries.

VIII. Polarimeters are used in the sugar industry for determining quality of both juice from sugar cane and the refined sucrose. Often, the sugar refineries use a modified polarimeter with a flow cell (and used in conjunction with a refractometer) called a saccharimeter.