CHAPTER 1
INTRODUCTION TO SPECTROPHOTOMETRY

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1.1 SPECTROPHOTOMETRY

Analytical chemistry is described as the area of chemistry responsible for characterizing the composition of matter, both qualitatively and quantitatively. Almost all chemists routinely make qualitative or quantitative measurements. The argument has been made that analytical chemistry is not a separate branch of chemistry, but simply the application of chemical knowledge [1]. In fact, probably have performed quantitative and qualitative analyses in other chemistry courses. For example, many introductory courses in chemistry include qualitative schemes for identifying inorganic ions and quantitative analyses involving titrations. As currently taught, the introductory course in analytical chemistry emphasizes quantitative (and sometimes qualitative) methods of analysis coupled with a heavy dose of equilibrium chemistry. Analytical chemistry, however, is more than equilibrium chemistry and a collection of analytical methods; it is an approach to solving chemical problems. The introductory analytical course is the ideal place in the chemistry curriculum to explore topics such as experimental design, sampling, calibration strategies, standardization, optimization, statistics, and the validation of experimental results. These topics are important in developing good experimental protocols, and in interpreting experimental results [2].

Newly emerging fields like pollution control, toxicology, food adulteration, forensic sciences, etc., demand simple, rapid and accurate methods for routine analysis. In the past few years, analysts have developed an extensive array of instrumental techniques. These techniques are extremely sensitive and can yield results rapidly to a high degree of accuracy. Among the instrumental analytical techniques, spectrophotometric technique occupies a unique position because of its simplicity, sensitivity, accuracy and rapidity. The availability of spectrophotometer made this technique indispensable to the modern analytical chemists [3]. Spectrophotometric methods for the determination of elements are based on the absorption of the visible and near ultraviolet radiation. Formerly, visible spectrophotometry was often called colorimetry and even now such definitions as colorimetric, photometric or absorptiometric methods are sometimes used in the literature, as equivalents to the term spectrophotometric method.
The basis of spectrophotometric method is the simple relationship between the absorption of radiation by a solution and the concentration of colored species in the solution. In order to determine a species spectrophotometrically it is usually converted in to a colored complex. The color of the determinant itself is utilized much less often. When the determinant is not colored, or forms no colored compounds, indirect spectrophotometric methods may be used for its determination. Spectrophotometric methods are remarkable for their versatility, sensitivity and precision. Almost all are direct and can be used for all the elements, except for the noble gases. A very extensive range of concentration may be covered, from macro quantities to traces ($10^{-2} – 10^{-8}$ %). Spectrophotometric methods are precise among the most of the instrumental methods of analysis [4]. It is the most important method for determining metals in alloys, minerals and complexes, owing to its selectivity. In comparison with atomic emission spectroscopy, atomic absorption spectroscopy and similar techniques, it offers the advantage of having calibration graphs that are linear over a wider range.

The act of identifying materials based on their color was probably one of the earliest examples of qualitative molecular absorption spectrophotometry. Also, the first recognition that color intensity can be the indicator of concentration was probably the earliest application of employing molecular absorption spectroscopy for quantitative determination. The first measurements were made by using the human eye as the detector and undispersed sunlight or artificial light as the light source. Later it was found that the accuracy and the precision could be improved by isolating specific frequencies of light using optical filters. Further improvement of the measurement came with the use of prism and grating monochromators for wavelength isolation. Photoelectric detectors were soon developed, but were quickly replaced with phototubes and photomultiplier tubes. The development of solid state microelectronics has now made available a wide range of detector type which are coupled with the computers, provide highly sophisticated readout electronic systems.

1.2 COLOR AND MOLECULAR STRUCTURE

Absorption spectrophotometry in the ultra-violet and visible regions is considered to be one of the valued techniques for the quantitative analysis. Visible
light represents a very small part of electromagnetic spectrum and is generally considered to extend from 380-780 nm. A solution or objects appears colored when it transmits or absorbs only part of the radiation in the visible spectrum. The optical characteristic of the substance is its absorption spectrum. There is a close relation between the color of a substance and its electronic structure [5,6]. A molecule exhibits absorption in the visible or ultraviolet range, when radiation causes an electronic transition, raising the molecule (ion) from the ground state to an excited state. The production or change of a color is connected with deformation of the normal electronic structure of the molecule. Irradiation causes variations in the electronic energy of the molecules containing one or more chromophoric groups, i.e. atomic groupings with unsaturated linkages.

Two or more chromophoric groups in the molecule often enhance one another’s effect, to deepen the color by displacing the absorption maximum ($\lambda_{\text{Max}}$) towards longer wavelengths (from the ultraviolet towards the red). This is called bathochromic shift. The displacement of the absorption maximum from the red towards the ultraviolet is known as a hypsochromic shift. The color of a molecule may be intensified by substituents called auxochromic groups. These groups may also affect bathochromic shifts. The color determining factor in a number of molecules is the introduction of conjugation of double bonds by means of electron donor and electron acceptor groups. The quantitative applicability of the absorption method is based on the fact that the number of photons absorbed is directly proportional to the number or concentration of atoms, ions or molecules [7]. As the pH is increased, spectrophotometric reagents ionize and their electronic structure becomes deformed, which often leads to a bathochromic shift of the absorption maximum. Ionization causes polarization of the chromophoric system. The formation of a chelate complex disturbs the electronic state of the organic molecule to produce, as a rule, a bathochromic shift, though some hypsochromic shifts are known. Most transition metals with an incomplete d-electron subshell have chromophoric properties. These metals may occur in various oxidation states. They can give color reactions with colorless reagents containing no chromophoric groups.
1.3 CALIBRATION CURVE

The common method of using the spectrophotometer requires the construction of a calibration curve for the constituents being determined. For this purpose, suitable quantities of the constituents are taken and treated in exactly the same way as the sample solution for development of the color, followed by the measurement of the absorption at the optimum wavelength. The absorbance is then plotted against concentration of the constituents. A straight line is obtained if Beer’s law is followed. This calibration curve may then be used in future determinations of the constituents under the same conditions. The calibration curve needs checking at intervals.

1.4 CHOICE OF THE WAVELENGTH

It is important to avoid making measurements in a region where the molar absorptivity (\( \varepsilon \)) changes rapidly with the wavelength. In such a region even a small error in setting the wavelength scale will result in a large change in the molar absorptivity [10]. Therefore, it is necessary to select the wavelength corresponding to the maximum of molar absorptivity. When the transmittance of the solution increases continuously over the wavelength range covered by the light filter, Beer’s law will not be obeyed.

1.5 SENSITIVITY OF SPECTROPHOTOMETRIC METHODS

Sensitivity is often described in terms of the molar absorptivity (\( \varepsilon, \text{ L mol}^{-1}\text{cm}^{-1} \)) of the metal ligand complex. The awareness of the sensitivity is very important in spectrophotometric determination of trace metals. The numerical expression [11-13] is the molar absorptivity (\( \varepsilon \)) at the wavelength of maximum absorbance of the colored species.

\[
\text{Molar absorptivity (\( \varepsilon \))} = \frac{A}{c \ell} \quad \text{-------- (1)}
\]

Sensitivity depends on the monochromaticity of the radiation. With monochromatic light of very narrow bandwidth corresponding to the wavelength of \( \lambda_{\text{max}} \), the maximum value of molar absorptivity is obtained.

Savvin [14] suggested a relation between sensitivity and molar absorptivity. He suggested the following criteria for describing the sensitivity.
Low sensitivity, \( \varepsilon < 2 \times 10^4 \), L mol\(^{-1}\) cm\(^{-1}\)

Moderate sensitivity \( \varepsilon = 2 - 6 \times 10^4 \), L mol\(^{-1}\) cm\(^{-1}\)

High sensitivity \( \varepsilon > 6 \times 10^4 \), L mol\(^{-1}\) cm\(^{-1}\)

It is generally stated [15] that the molar absorptivity will not exceed approximately \( 10^5 \).

Other way of specifying sensitivity are as specific absorptivity [16] or the Sandell’s sensitivity [17]; both methods give the sensitivity in terms of mass of analyte per unit volume of solution. Such an approach is perhaps more convenient than using molar absorptivities as a basis of comparison. The Sandell’s sensitivity is the concentration of the analyte (in \( \mu \text{g mL}^{-1} \)) which will give an absorbance of 0.001 in a cell of path length 1 cm and is expressed as \( \mu \text{g cm}^{-2} \). Organic reagents with high molecular weights furnish maximum sensitivity if used as chromogenic agents. Detection limits can be reduced to somewhat by solvent selection because molar absorptivities depend on the solvent system. Another technique used to increase the detection limit is to use indirect determinations, where a stoichiometric gain in the number of chromophores may result or the newly formed chromophore may have a higher molar absorptivity. Reaction rate methods can sometimes have lower detection limits than do conventional spectrophotometric measurements.

1.6 PRECISION AND ACCURACY

Precision describes the reproducibility of results where accuracy denotes the nearness of a measurement to its accepted value. The accuracy and precision of spectrophotometric method depends on three major factors. Instrumental limitations, chemical variables and operator’s skill. Instrumental limitations are often determined by the quality of the instruments, optical, mechanical and electronic systems. Chemical variables are determined by purity of standards, reagents and chromophore stability, reaction rates, reaction stoichiometry, pH and temperature control. These factors are usually determined by the methodology chosen for the analysis. Under ideal conditions it is possible to achieve relative standard deviations in concentrations as low as about 0.5%. This enables the determination of microquantities of components. The precision of spectrophotometric method also depends on concentration of the determinant.
Visual methods generally give results with a precision of 1–10%. The precision of the photometric method is of course, higher and varies from 0.5–2% under suitable measuring conditions.

The precision attainable is a function of the absorbance measured. The error observed is, as expected, very large on lower side of concentrations. When intensely colored solutions are measured, only an insignificant part of the radiation is transmitted and on the logarithmic absorbance scale the gradations are so close that the reading error is very high. Precision is conveniently expressed in terms of the average deviation from the mean or in terms of standard deviation. When applied to small sets of data with which the analytical chemists work, the standard deviation is the most reliable estimate of the indeterminate uncertainty. When the standard deviation turns out to be approximately proportional to the amount present in the formation on the precision can be expressed in percent by using the coefficient of variation. Mathematical equation for the calculation of coefficient of variation is given below

\[ CV = \left( \frac{\sigma}{\bar{x}} \times 100 \right) \]

Where \( \sigma = \) Standard deviation and \( \bar{x} = \) Arithmetic mean of a series of measurements.

1.6.1 Detection Limit

Detection limit is the smallest concentration of a solution of an element that can be detected with 95% certainty [18,19]. This is the quantity of the element that gives a reading equal to twice the standard deviation of a series of any least ten determinations taken with solutions of concentrations which are close to the level of the blank. Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Based on the standard deviation of the reagent blank and the slope of the calibration curve of the analyte, the detection limit (\( D_L \)) may be expressed as:

\[ D_L = \frac{3.3 \cdot \sigma}{S} \]

Where \( \sigma = \) the standard deviation of the reagent blank
\( S \) = the slope of the calibration curve

The slope \( S \) may be estimated from the calibration curve of the analyte. The estimate of \( \sigma \) may be measured based on the standard deviation of the reagent blank.

### 1.6.2 Quantitation Limit

The quantitation limit is generally determined by the analysis of samples with known concentrations of analyte with those of blank samples and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision [20,21]. Based on the standard deviation of the reagent blank samples and the slope of the calibration curve of the analyte, the quantitation limit \( (Q_L) \) may be expressed as:

\[
Q_L = \frac{10 \sigma}{S} \quad (4)
\]

where \( \sigma \) = the standard deviation of the reagent blank

\( S \) = the slope of the calibration curve

The slope \( S \) may be estimated from the calibration curve of the analyte. The estimate of \( \sigma \) may be measured based on the standard deviation of the reagent blank.

### 1.6.3 Comparison of the Results

The comparison of the values obtained from a set of results with either (a) the true value or (b) other sets of data makes it possible to determine whether the analytical procedure has been accurate and/or precise, or if it is superior to another method. There are two common methods for comparing results: (a) Student’s \( t \)-test and (b) the variance ratio test (F-test) [22,23].

These methods of test require knowledge of what is known as the number of degrees of freedom. In statistical terms this is the number of independent values necessary to determine the statistical quantity. Thus a sample of \( n \) values has \( n \) degrees of freedom, whilst the sum \( \sum (x - \bar{x})^2 \) is considered to have \( n-1 \) degrees of freedom, as for any defined value of \( \bar{x} \) only \( n-1 \) values can be freely assigned, the \( n^{th} \) being automatically defined from the other values.
(a) Student’s t-test

This is a test used for small samples; its purpose is to compare the mean from a sample with some standard values and to express some level of confidence in the significance of the comparison. It is also used to test the difference between the means of the two sets of data.

\[ t = \frac{\overline{x} - \mu}{\frac{s}{\sqrt{n}}} \]  

Where \( s \) = Standard deviation, \( \overline{x} \) = Arithmetic mean of a series of measurements, ‘\( \mu \)’ is the true value and ‘\( n \)’ is the number of trials of the measurements.

It is then related to a set of \( t \)-tables [22] in which the probability of the \( t \)-value falling within certain limits is expressed, either as a percentage or as a function of unity, relative to the number of degrees of freedom.

(b) The Variance Ratio Test (F-test)

This is used to compare the precisions of two sets of data of two different analytical methods or the results from two different laboratories. It is calculated from the following equation [22, 23]:

\[ F = \frac{s_A^2}{s_B^2} \]  

The larger value of \( s \) is always used as the numerator so that the value of \( F \) is always greater than unity. The value obtained for \( F \) is then checked for its significance against values in the \( F \)– table calculated from an \( F \)– distribution [22] corresponding to the numbers of degrees of freedom for the two sets of data.

1.6.4 Comparison of the Means of Two Samples

When a new analytical method is being developed it is usual practice to compare the values of the mean and precision of the new (proposed) method with those of an established (reference) procedure [23].

There are two common methods for comparing results: (a) t-test and (b) the variance test (F-test).
(a) t – Test

This method is also used to compare the values of the mean and precision of the test method with those of the reference methods [22,23]. The value of ‘t’ when comparing two sample means $\bar{x}_1$ and $\bar{x}_2$ is given by the expression:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{S_p \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \quad \text{---------------- (7)}$$

Where $S_p$ is the pool standard deviation, is calculated from the two samples standard deviations ‘$s_1$’ and ‘$s_2$’ as follows:

$$S_p = \left(\frac{(n_1 - 1) S_1^2 + (n_2 - 1) S_2^2}{n_1 + n_2 - 2}\right)^{\frac{1}{2}} \quad \text{---------------- (8)}$$

‘$n_1$’ and ‘$n_2$’ are the number of trials of first and second method.

(b) Variance Ratio Test ($F$ – Test)

The $F$-test must also be applied to establish that there is no significant difference between the precision of the two methods. The value of $F$ calculated from equation (6). The larger value of $s$ is always used as the numerator so that the value of $F$ is always greater than unity. The value obtained for $F$ is then checked for its significance against values in the $F$ – table calculated from an $F$ – distribution [22] corresponding to the numbers of degrees of freedom for the two sets of data. Thus, the calculated value of $F$ is less than the tabulated value, therefore the method have comparable precisions (standard deviations).

1.7 COLOR DEVELOPMENT

There are only a few elements, which give sufficiently intense absorption by themselves and are spectrophotometrically measurable. Majority of the substances are generally determined indirectly in a variety of ways, such as

i. Substances may be converted by a suitable reagent to an absorbing product changing oxidation state to a colored valence state

ii. Adding complexing agent to get colored complexes and so on.

iii. Organic complexing agents are found to be more selective and sensitive color developing agents.
1.7.1 Requirements of a Color Developer

A color developer should possess a high molar absorptivity, high selectivity and the spectrum of the complex should be significantly different from that of the reagent.

1.7.2 The Criteria for Satisfactory Spectrophotometric Analysis

Though spectrophotometric methods are versatile in nature, in order to have successful and satisfactory results, the process of analysis needs careful operations. Since the color development in spectrophotometry involves diverse type of reactions, a number of points need to be ensured before applying the method for a particular application. Some of the points have to be considered are discussed in the following sections.

1.7.2.1 Specificity of the color reactions

Very few reactions are specific for a particular substance, but may give colors for a small group of related substances only. Because of this selective character of many colorimetric reactions, it is important to control the operational procedure so that the color is specific for the component being determined. This may be achieved by isolating the substance by the normal methods of inorganic analysis. But these separation methods are often tedious and time consuming. Further there is every possibility of appreciable loss of the analyte during these separations.

The specificity in colorimetric reactions can be achieved by introducing other complex forming compounds. These are required to suppress the action of interfering substance by formation of complex ions or of non-reactive complexes. When the colorimetric reaction takes place within well-defined limits of pH, adjustment of pH may also sometimes help to achieve the desired specificity in certain cases. The methods of selective absorption, chromatographic separations and ion exchange separations are also of use in certain cases. Solvent extraction method also finds its application in achieving specificity in the spectrophotometric determinations. The interfering substances are removed by extraction with an organic solvent, sometimes after suitable chemical treatment. Alternatively the substance to be determined can also be isolated from the interfering species by
converting it into an organic complex, which is then selectively extracted into a suitable organic solvent.

1.7.2.2 Proportionality between color and concentration

For colorimeters, it is important that color intensity should increase linearly with concentration of the compound to be determined. This is not necessary for photoelectric colorimeters or spectrophotometers. Since a calibration curve may be constructed relating the instrumental reading of the color with the concentration of the solution. It is desirable that the system follows Beer’s law even when photoelectric colorimeters are used.

1.7.2.3 Stability of the color and clarity of the solutions

The color produced must be stable so as to allow accurate readings to be taken. The period over which maximum absorbance remains constant must be long enough for precise measurement to be made. Stability of the color is influenced by experimental conditions like temperature, pH etc. The solution must be free from precipitate if comparison is to be made with a clear standard. Turbidity scatters as well as absorbs the light.

1.7.2.4 Reproducibility and sensitivity

The colorimetric procedure must give reproducible results under specific experimental conditions. The reaction need not necessarily represent a stoichiometrically quantitative chemical change.

It is desirable, particularly when minute amounts of substances are to be determined, that the color reactions be highly sensitive. It is also desirable that the reaction product absorbs strongly in the visible rather than in the ultraviolet region, as the interfering effect of other substances is usually more pronounced in the ultraviolet region.

1.8 CHOICE OF SOLVENT

The solvent, which is to be used in colorimetric or spectrophotometric determinations, must meet certain requirements. It must be a good solvent for the substance under determination. Before using a particular solvent, it must be
ensured that it does not interact with the solute. The solvent must not show significant absorption at the wavelength to be employed in the determination.

For inorganic compounds, water normally meets these requirements, but for majority of organic compounds, it is necessary to use an organic solvent. All solvents show absorption at some point in the ultraviolet region and care must be taken to choose a solvent for a particular determination which does not absorb in the requisite wavelength region. Any impurities present in the solvents may affect the absorption at certain wavelength and it is therefore, essential to employ materials of the highest purity.

1.9 LIMITATIONS

The common but unrecognized problem in measuring the absorbance is stray light error. All wavelength isolation devices tend to produce some low intensity radiations at wavelengths other than the desired one. This is usually due to the optical imperfections, or simply from scattered light due to dust particles on optical surface. Because one has usually selected a wavelength at which the compound of interest absorbs most strongly, the stray light falling on the sample is of wavelengths at which the compound does not absorb strongly. Thus the stray light errors will result in a negative bias for absorbance readings which can be represented in the equation

\[ T_{\text{obs}} = \frac{T_{\text{true}} + \rho}{1 + \rho} \quad \text{--------- (9)} \]

Where \( \rho \) is the fraction of all the light coming from the wavelength isolation device, which is stray light, and \( T_{\text{obs}} \) and \( T_{\text{true}} \) are the observed and true transmittances, respectively. Normally the absolute amount of stray light tends to be relatively constant with respect to the wavelength. But the fraction of stray light is highly wavelength dependent because the amount of energy of the selected wavelength depends on the source intensity at that wavelength. Thus, stray light errors are most predominant at long and short wavelengths and when high absorbance is measured.

A common error is encountered when making the measurements is called finite slit width effect. The exit slit of the monochromator subtends a portion of the
dispersed continuum from the grating or prism. If any light is to pass through the slit it must have a finite width. However, due to its width, more than one wavelength of light, called the bandwidth, emerges. Although most of the energy emerging from the slit is of selected or nominal wavelength, a small percentage is of adjacent wavelengths, called spectral bandwidth. This is simply the wavelength span, centered on the nominal wavelength, containing 75% of the radiant energy emerging from the slit. Thus, the narrower the spectral band width, the better conformity to Beer’s law. If the spectral bandwidth is too wide, negative deviation from the Beer’s law occurs, resulting in a false absorbance reading.

Errors also occur when a distilled water blank is used instead of a true blank for 100% transmittance or baseline reading. Even though there are no known absorbing species in distilled water as well as in the blank reagent solution, the difference in the refractive indices between the sample solution and the reference solution must be kept reasonably close or reflective loses at the cell windows may not be the same. Even when the incident light is highly collimated and falls on the cell window at normal incidence, a small fraction of the light is reflected back at each interface where there is a refractive index difference, at the two air-window interfaces, and the two window-solution interfaces. Because the sample and the reference cells are of the same composition, reflections from the air-window interfaces are compensated for. However, reflections from the solution-window interfaces may be different if the refractive indices of the sample and the blank are not nearly the same.

1.10 APPLICATIONS

The greatest use of spectrophotometry lies in its application to quantitative measurements. The reasons for this stem from the ease with which most spectrophotometric measurements can be made, their sensitivity and precision, and the relatively low cost of instrument purchase and operation. A variety of techniques have been developed for different types of samples. Direct determinations are made when the analyte molecule contains a chromophore, thus allowing the direct measurement of its absorbance. Standards must be used to determine the absorptivity so that concentration can be calculated by using the equations or by establishing a calibration plot from which the concentration can be
determined by graphic interpretation or by regression analysis. Indirect determinations are commonly used when the analyte molecule does not contain a suitable chromophore. In these instances the analyte is made to quantitatively react with a molecules containing a chromophore and correlating the diminution of absorbance with the concentration of the analyte or by reacting with a reagent, which produces a chromophoric groups. Spectrophotometric analysis continues to be one of the most widely used analytical techniques available. Many methods are available for a variety of analytes (such as colored, colorless, natural, synthetic, inorganic and organic analytes) and sample types ranging from in-situ biological assays to the determination of trace elements in steels. Many medical diagnostic test kits use photometric measurements. Diabetics commonly use blood-glucose analysis kits based on the glucose oxidase enzyme reaction that secondarily produces a colored product. In the food industry, winemakers have long recognized the effect of iron levels on the taste of wines and consequently are one the largest users of 1,10-phenanthroline for determining iron spectrophotometrically. A common field test for chlorine in swimming pools and drinking water is based on the color produced by the action of chlorine on o-tolidine.

Many compilations of methodology for a variety of analytes and sample types that are regularly updated are available [24-26]. Other general sources for spectrophotometric analysis are commonly consulted and found helpful [27, 28]. Methods specific for metals [29], and nonmetals [30] should be consulted when dealing with these analytes. Standard methods specific to certain industries and areas of study are very useful sources when specific sample types are being considered, such as water and waste water [31] and pharmaceuticals [32].

1.11 PRESENT INVESTIGATION

The work presented in chapter-2-9 presents spectrophotometric determination of some bioactive compounds. Chapter 2 describes spectrophotometric determination of nitrite by diazotization method. Chapter 3 describes the determination of hydroxylamine and its derivatives in pharmaceuticals using spectrophotometry. Chapter 4 & 5 describes new reagents for the spectrophotometric determination of chromium and hypochlorite. Chapter
describes spectrophotometric determination of selenium in environmental samples using resorcinol, chloroacetyl catechol and 4-chloro-2-nitrophenol as coupling reagents. **Chapter 7-9** deals with the spectrophotometric determination of some drugs in pharmaceuticals. **Chapter 10** deals with the synthesis and single crystal x-ray crystallographic studies on few chalcones. The following reagents are used in this study.

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<th>Xylene cyanol FF</th>
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<th>Citalopram hydrobromide</th>
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1.12 REFERENCES


