Benha University
Faculty of Agriculture
Biochemistry Department

Instrumental Practical Part

(Chemistry 5)

Biotechnology Program (Level 2)

(Section 1)

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Instrumental (section 1)

Spectrophotometry

Spectrophotometry is a method to measure how much a chemical substance absorbs light by measuring the intensity of light as a beam of light passes through sample solution. The basic principle is that each compound absorbs or transmits light over a certain range of wavelength. This measurement can also be used to measure the amount of a known chemical substance. Spectrophotometry is one of the most useful methods of quantitative analysis in various fields such as chemistry, physics, biochemistry, material and chemical engineering and clinical applications.

Every chemical compound absorbs, transmits, or reflects light (electromagnetic radiation) over a certain range of wavelength. Spectrophotometry is a measurement of how much a chemical substance absorbs or transmits. Spectrophotometry is widely used for quantitative analysis in various areas (e.g., chemistry, physics, biology, biochemistry, material and chemical engineering, clinical applications, industrial applications, etc). Any application that deals with chemical substances or materials can use this technique. In biochemistry, for example, it is used to determine enzyme-catalyzed reactions. In clinical applications, it is used to examine blood or tissues for clinical diagnosis. There are also several variations of the spectrophotometry such as atomic absorption spectrophotometry and atomic emission spectrophotometry.

A spectrophotometer is an instrument that measures the amount of photons (the intensity of light) absorbed after it passes through sample solution. With the spectrophotometer, the amount of a known chemical substance (concentrations) can also be determined by measuring the intensity of light detected. Depending on the range of wavelength of light source, it can be classified into two different types:

- **UV-visible spectrophotometer**: uses light over the ultraviolet range (185 - 400 nm) and visible range (400 - 700 nm) of electromagnetic radiation spectrum.
- **IR spectrophotometer**: uses light over the infrared range (700 - 15000 nm) of electromagnetic radiation spectrum.

In visible spectrophotometry, the absorption or the transmission of a certain substance can be determined by the observed color. For instance, a solution sample that absorbs light over all visible ranges (i.e., transmits none of visible wavelengths) appears black in theory. On the other hand, if all visible wavelengths are transmitted (i.e., absorbs nothing), the solution sample appears white. If a solution sample absorbs red light (~700 nm), it appears green because green is the complementary color of red. Visible spectrophotometers, in practice, use a prism to narrow down a certain range of wavelength (to filter out other wavelengths) so that the particular beam of light is passed through a solution sample.

Devices and mechanism

Figure 1 illustrates the basic structure of spectrophotometers. It consists of a light source, a collimator, a monochromator, a wavelength selector, a cuvette for sample solution, a photoelectric detector, and a digital display or a meter. Detailed mechanism is described below. Figure 2 shows a sample spectrophotometer (Model: Spectronic 20D).

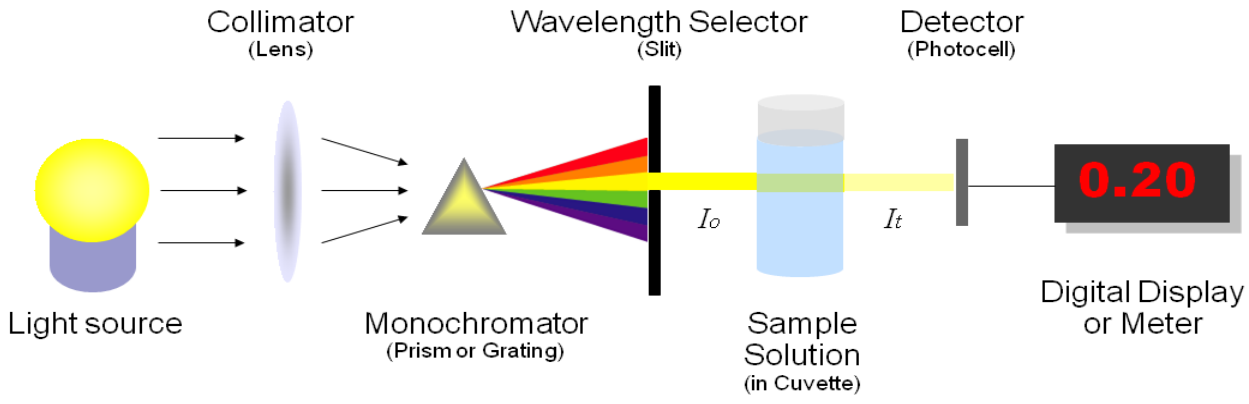

Figure 1: Basic structure of spectrophotometers (illustrated by Heesung Shim)

A spectrophotometer, in general, consists of two devices; a spectrometer and a photometer. A spectrometer is a device that produces, typically disperses and measures light. A photometer indicates the photoelectric detector that measures the intensity of light.

- **Spectrometer**: It produces a desired range of wavelength of light. First a collimator (lens) transmits a straight beam of light (photons) that passes through a monochromator (prism) to split it into several component wavelengths (spectrum). Then a wavelength selector (slit) transmits only the desired wavelengths, as shown in Figure 1.
- **Photometer**: After the desired range of wavelength of light passes through the solution of a sample in cuvette, the photometer detects the amount of photons that is absorbed and then sends a signal to a galvanometer or a digital display, as illustrated in Figure 1.

Applications of Differential Spectrophotometry in Analytical Chemistry

Differential spectroscopy is an elegant and powerful analytical method, based on the relatively simple principles of classical absorption spectroscopy. Some background to differential spectroscopy as well as an updated applications in the period 2000–2013 are reviewed. Associated with chemometrics, it can become a powerful tool for the pharmaceutical industry. Indeed, it is suitable for analysis of solid, liquid, and biotechnological pharmaceutical forms. This review focuses on applications used for qualitative and quantitative analysis. Moreover, it can be implemented during pharmaceutical development, in production for process monitoring, and in quality control laboratories.

The basis of early spectroscopic measurements and many present quantitative and qualitative analytical methods is Lambert-Beer's law, often also referred as the Bouguer Lambert law. The law was presented in various forms by Pierre Bouguer (1729), Johann H. Lambert (1760), and August Beer (1852). Bouguer first explained that "in a medium of uniform transparency the light remaining in a collimated beam is an exponential function of the length of the path in the medium" (1729, pp. 16–22).

A variety of spectroscopic techniques make use of the absorption of electromagnetic radiation by matter when a beam of light passes through a volume of length *L*,

containing a sample of concentration *c*, and at the end of the light path the intensity is measured by a suitable detector, as can be seen in Figure 1

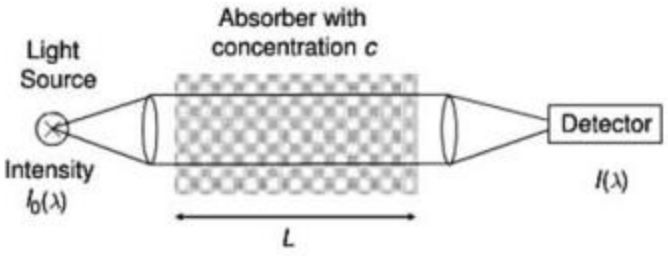

FIG. 1. The basic principle of absorption spectroscopy detection

For the determination of a particular species, it would in principle be necessary to quantify all other factors influencing the intensity of radiation. In the laboratory, this can be achieved by removing the sample from the light path. Quantitative analyses often involve the spectrophotometric resolution of mixtures of two components with partly overlapping spectra, and the greater the extent of overlap, the more difficult the resolution. Not surprisingly, this topic has been the subject of a number of chemometric studies originally intended for the resolution of binary mixtures and later extended to three or more components (Brown et al., 1988; Hargis and Howell, 1988).

APPLICATIONS

Analytical applications of differential spectrophotometry are connected mainly with the determination of larger concentrations of metals. There are, however, methods available for the determination of anions of organic substances. Some methods used in infrared analysis similar in technique to differential spectrophotometry will be also presented herein. In the analysis of mixtures where bands of individual substances are weakly separated, the method of spectral differentiation using a spectrophotometer equipped with a special electronic system that makes it possible to obtain such a spectrum by an ordinary absorbance measurement is used more and more frequently. Secondary spectra are used to rapidly determine the compound identity, e.g., isoenzymes of pyruvate kinase, and in determining the differences in protein composition (Greenfield, 1999). They have also been successfully used to determine drugs in pharmaceutical preparations, plasma, and serum. Furthermore, some of the most important pharmaceutical, food, clinical, and environmental applications of the technique in the above-mentioned period are discussed as follows.

Pharmaceutical Analysis

Rapid and accurate binary mixture resolution of ephedrine hydrochloride and theophylline was performed (Erk, 2000a). Differential-derivative spectrophotometry with a zero-crossing measurement technique was used for the quantitative determination of ephedrine hydrochloride and theophylline in pharmaceuticals without prior sample pretreatment or separation. Commercial tablet and laboratory-prepared mixtures containing both drugs were assayed using the developed method with good precision and accuracy.

Clinical Analysis

Eliamid is a secondary metabolite isolated from two bacterial strains. This molecule features a linear polyketide backbone terminated by a tetramic acid amide moiety. Eliamid shows high and specific cytostatic action on human lymphoma and cervix carcinoma cell lines. The isolation, biological activity evaluation, structure elucidation, total synthesis, and studies on the biosynthesis of eliamid, a potent complex I inhibitor with some other interesting activities, were presented by Hofle et al.¨ (2012). Differential spectroscopy experiments with beef heart submitocondrial particles established that eliamid is a potent inhibitor of the NADH-ubiquinone oxidoreductase complex

Food Analysis

A differential kinetic spectrophotometric method was researched for the simultaneous determination of iron and aluminum in food samples. It was based on the direct reaction kinetics and spectrophotometry of these two metal ions with chrome azurol S (CAS) in ethylenediamine-hydrochloric acid buffer (pH 6.3) (Ni et al., 2007). The proposed method was applied for the determination of iron and aluminum in some commercial food samples with satisfactory results

Environmental Analysis

A procedure for the simultaneous kinetic spectrophotometric determination of aminocarb and carbaryl in vegetable and water samples was described (Ni et al., 2009a). The method was based on the differential oxidation rates of aminocarb and carbaryl when they are reacted with the oxidant, potassium ferricyanide ($K_3Fe(CN)_6$), in an appropriate alkaline medium. Under the optimum experimental conditions, the linear ranges were 0.05–0.6 mg L^{-1} and 0.1–1.2 mg L^{-1} for

aminocarb and carbaryl, respectively. The kinetic data collected were well processed by chemometrics methods, i.e., PLS and PC-RBF-ANN calibration models gave the lowest prediction errors. Dichlorprop (2,4-dichlorophenoxy-2-propionic acid) was shown to be enantioselectively transformed in surface soil after application to an experimental field. The (*S*)-enantiomer had a half-life of about 4 days while the (*R*)-enantiomer had a half-life of about eight days. The enantioselective interaction between *Penicillium expansum* alkaline lipase and chiral phenoxypropionic acid herbicide dichlorprop was studied using UV differential spectrophotometry and fluorescence spectrophotometry in a phosphate buffer solution of pH 8 (Wen et al., 2009). Chiral differences in the UV absorption and fluorescence spectra of lipase with dichlorprop were detected. It is noteworthy that chiral pesticides applied to the environment can alter the activity and conformation of enzymes, consequently influencing their function and physical metabolism. Most environmental research on chiral pesticides has thus far been focused on investigating the enantioselectivity of degradation and toxicity.

Steps in developing a spectrophotometric analytical method:

1- The instrument must be warmed for 15 min.
2- Set a monochromatic wavelength for the maximum absorption wavelength.
3- Wipe the tube containing the blank solution with a lab wipe.
4- Place it into sample holder. Close cover
5- Set zero absorbance.
6- Remove blank tube, wipe off the cuvette of sample and insert it.
7- Read and record the absorbance.
8- Calculate the concentration of the sample using Beer Lambert equation:
$A = \varepsilon CL$.

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