7 chapter

Ultraviolet, Visible, and Fluorescence Spectroscopy

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7.1 INTRODUCTION

Spectroscopy in the ultraviolet-visible (UV-Vis) range is one of the most commonly encountered laboratory techniques in food analysis. Diverse examples, such as the quantification of macrocomponents (total carbohydrate by the phenol-sulfuric acid method), quantification of microcomponents (thiamine by the thiochrome fluorometric procedure), estimates of rancidity (lipid oxidation status by the thiobarbituric acid test), and surveillance testing (enzyme-linked immunoassays), are presented in this text. In each of these cases, the analytical signal for which the assay is based is either the emission or absorption of radiation in the UV-Vis range. This signal may be inherent in the analyte, such as the absorbance of radiation in the visible range by pigments, or a result of a chemical reaction involving the analyte, such as the colorimetric copper-based Lowry method for the analysis of soluble protein.

Electromagnetic radiation in the UV-Vis portion of the spectrum ranges in wavelength from approximately 200–700 nm. The accessible **UV range** for common laboratory analyses runs from 200 to 350 nm and the **Vis range** from 350 to 700 nm (Table 7.1). The UV range is colorless to the human eye, while different wavelengths in the visible range each have a characteristic color, ranging from violet at the short wavelength end of the spectrum to red at the long wavelength end of the spectrum. Spectroscopy utilizing radiation in the UV-Vis range may be divided into two general categories, **absorbance** and **fluorescence** spectroscopies, based on the type of radiation-matter interaction that is being monitored. Each of these two types of spectroscopy may be subdivided further into **qualitative** and **quantitative** techniques. In general, quantitative absorption spectroscopy is the most common of the subdivisions within UV-Vis spectroscopy.

7.1 table Spectrum of visible radiation

Wavelength (nm)	*Color*	*Complementary hue*[a]
<380	Ultraviolet	
380–420	Violet	Yellow-green
420–440	Violet-blue	Yellow
440–470	Blue	Orange
470–500	Blue-green	Red
500–520	Green	Purple
520–550	Yellow-green	Violet
550–580	Yellow	Violet-blue
580–620	Orange	Blue
620–680	Red	Blue-green
680–780	Purple	Green
>780	Near infrared	

[a]Complementary hue refers to the color observed for a solution that shows maximum absorbance at the designated wavelength assuming a continuous spectrum "white" light source

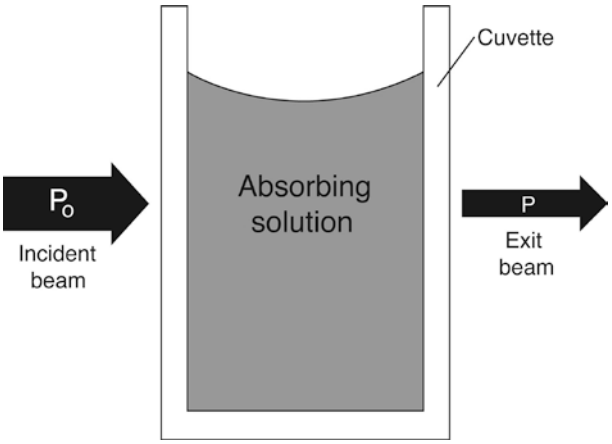
7.2 ULTRAVIOLET AND VISIBLE ABSORPTION SPECTROSCOPY

7.2.1 Basis of Quantitative Absorption Spectroscopy

The objective of quantitative absorption spectroscopy is to determine the concentration of analyte in a given sample solution. The determination is based on the measurement of the amount of light absorbed from a reference beam as it passes through the sample solution. In some cases the analyte may naturally absorb radiation in the UV-Vis range, such that the chemical nature of the analyte is not modified during the analysis. In other cases analytes that do not absorb radiation in the UV-Vis range are chemically modified during the analysis, converting them to a species that absorbs radiation of the appropriate wavelength. In either case the presence of analyte in the solution will affect the amount of radiation transmitted through the solution, and, hence, the relative transmittance or absorbance of the solution may be used as an index of analyte concentration.

In actual practice, the solution to be analyzed is contained in an absorption cell and placed in the path of radiation of a selected wavelength(s). The amount of radiation passing through the sample is then measured relative to a reference sample. The relative amount of light passing through the sample is then used to estimate the analyte concentration. The process of absorption may be depicted as in Fig. 7.1. The radiation incident on the absorption cell, P_0, will have significantly greater radiant power than the radiation exiting the opposite side of the cell, P. The decrease in radiant power as the beam passes through the solution is due to the capture (absorption) of photons by the absorbing species. The relationship between the power of the incident and exiting beams typically is expressed in terms of either the transmittance or the absorbance of the solution. The **transmittance** (T) of a solution is defined as the ratio of P to P_0 as given in Eq. 7.1. Transmittance also may be expressed as a percentage as given in Eq. 7.2.

$$T = P / P_0 \tag{7.1}$$



7.1 figure Attenuation of a beam of radiation as it passes through a cuvette containing an absorbing solution

$$\%T = \left(P / P_0\right) \times 100 \tag{7.2}$$

where:

T = transmittance
P_0 = radiant power of beam incident on absorption cell
P = radiant power of beam exiting the absorption cell
$\%T$ = percent transmittance

The terms T and $\%T$ are intuitively appealing, as they express the fraction of the incident light absorbed by the solution. However, T and $\%T$ are not directly proportional to the concentration of the absorbing analyte in the sample solution. The nonlinear relationship between transmittance and concentration is an inconvenience since analysts are generally interested in analyte concentrations. A second term used to describe the relationship between P and P_0 is **absorbance** (A). Absorbance is defined with respect to T as shown in Eq. 7.3.

$$A = \log\left(P_0 / P\right) = -\log T = 2 - \log \%T \tag{7.3}$$

where:

A = absorbance
T and $\%T$ = as in Eqs. 7.1 and 7.2, respectively

Absorbance is a convenient expression in that, under appropriate conditions, it is directly proportional to the concentration of the absorbing species in the solution. Note that based on these definitions for A and T, the absorbance of a solution *is not* simply unity minus the transmittance. In quantitative spectroscopy, the fraction of the incident beam that is not transmitted does not equal the solution's absorbance (A).

The relationship between the absorbance of a solution and the concentration of the absorbing species is known as **Beer's law** (Eq. 7.4).

$$A = abc \tag{7.4}$$

where:

A = absorbance
c = concentration of absorbing species
b = pathlength through solution (cm)
a = absorptivity

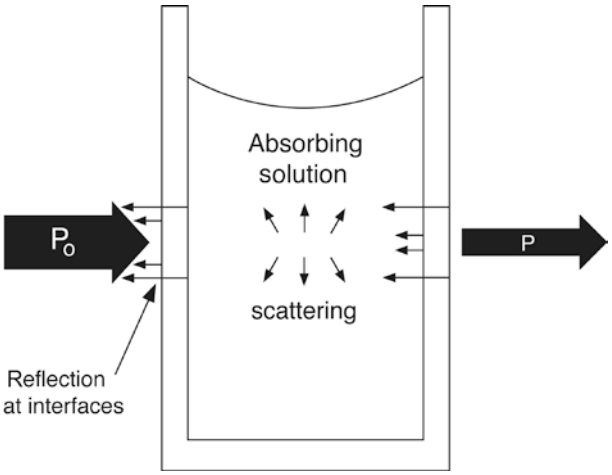
There are no units associated with absorbance, A, since it is the log of a ratio of beam powers. The concentration term, c, may be expressed in any appropriate units (M, mM, mg/mL, %). The pathlength, b, is in units of cm. The **absorptivity**, a, of a given species is a proportionality constant dependent on the molecular properties of the species. The absorptivity is wavelength dependent and may vary depending on the chemical environment (pH, ionic strength, solvent, etc.) the absorbing species is experiencing. The units of the absorptivity term are $(\text{cm})^{-1}$ $(\text{concentration})^{-1}$. In the special case where the concentration of the analyte is reported in units of molarity, the absorptivity term has units of $(\text{cm})^{-1}$ $(M)^{-1}$. Under these conditions, it is designated by the symbol ε, which is referred to as the **molar absorption coefficient**. Beer's law expressed in terms of the molar absorption coefficient is given in Eq. 7.5. In this case, c refers specifically to the molar concentration of the analyte:

$$A = \varepsilon bc \tag{7.5}$$

where:

A and b = as in Eq. 7.4
ε = molar absorption coefficient
c = concentration in units of molarity

Quantitative spectroscopy is dependent on the analyst being able to accurately measure the fraction of an incident light beam that is absorbed by the analyte in a given solution. This apparently simple task is somewhat complicated in actual practice due to processes other than analyte absorption that also result in significant decreases in the power of the incident beam. A pictorial summary of reflection and scattering processes that will decrease the power of an incident beam is given in Fig. 7.2. It is clear that these processes must be accounted for if a truly quantitative estimate of analyte absorption is necessary. In practice, a reference cell is used to correct for these processes. A **reference cell** is one that, in theory, exactly matches the sample absorption cell with the exception that it contains no analyte. Reference cells are often prepared by filling appropriate absorption cells with water. The reference cell is placed in the path of the light beam, and the power of the radiation exiting the reference cell is



7.2 figure Factors contributing to the attenuation of a beam of radiation as it passes through a cuvette containing an absorbing solution

measured and taken as P_o for the sample cell. This procedure assumes that all processes except the selective absorption of radiation by the analyte are equivalent for the sample and reference cells. The absorbance actually measured in the laboratory approximates Eq. 7.6.

$$A = \log\left(P_{\text{solvent}} / P_{\text{analyte solution}}\right) \cong \log\left(P_0 / P\right) \quad (7.6)$$

where:

P_{solvent} = radiant power of beam exiting cell containing solvent (blank)
$P_{\text{analyte solution}}$ = radiant power of beam exiting cell containing analyte solution
P_0 and P = as in Eq. 7.1.
A as in Eq. 7.3.

7.2.2 Deviations from Beer's Law

It should never be assumed that Beer's law is strictly obeyed. Indeed, there are several reasons for which the predicted linear relationship between absorbance and concentration may not be observed. In general, Beer's law is applicable only to dilute solutions, up to approximately 10 m*M* for most analytes. The actual concentration at which the law becomes limiting will depend on the chemistry of the analyte. As analyte concentrations increase, the intermolecular distances in a given sample solution will decrease, eventually reaching a point at which neighboring molecules mutually affect the charge distribution of the other. This perturbation may significantly affect the ability of the analyte to capture photons of a given wavelength; that is, it may alter the analyte's absorptivity (a). This causes the linear relationship between concentration and absorption to break down since the absorptivity term is the constant of proportionality in Beer's law (assuming a constant pathlength, b). Other chemical processes also may result in deviations from Beer's law, such as the reversible association-dissociation of analyte molecules or the ionization of a weak acid in an unbuffered solvent. In each of these cases, the predominant form of the analyte may change as the concentration is varied. If the different forms of the analyte (e.g., ionized versus neutral) have different absorptivities (a), then a linear relationship between concentration and absorbance will not be observed.

A further source of deviation from Beer's law may arise from limitations in the instrumentation used for absorbance measurements. Beer's law strictly applies to situations in which the radiation passing through the sample is monochromatic, since under these conditions a single absorptivity value describes the interaction of the analyte with all the radiation passing through the sample. If the radiation passing through a sample is polychromatic and there is variability in the absorptivity constants for the different constituent wavelengths, then Beer's law will not be obeyed. An extreme example of this behavior occurs when radiation of the ideal wavelength and stray radiation of a wavelength that is not absorbed at all by the analyte simultaneously pass through the sample to the detector. In this case, the observed transmittance will be defined as in Eq. 7.7. Note that a limiting absorbance value will be reached as $Ps >> P$, which will occur at relatively high concentrations of the analyte:

$$A = \log\left(P_0 + P_s\right) / \left(P + P_s\right) \quad (7.7)$$

where:

P_s = radiant power of stray light
A = as in Eq. 7.3
P and P_0 = as in Eq. 7.1.

7.2.3 Procedural Considerations

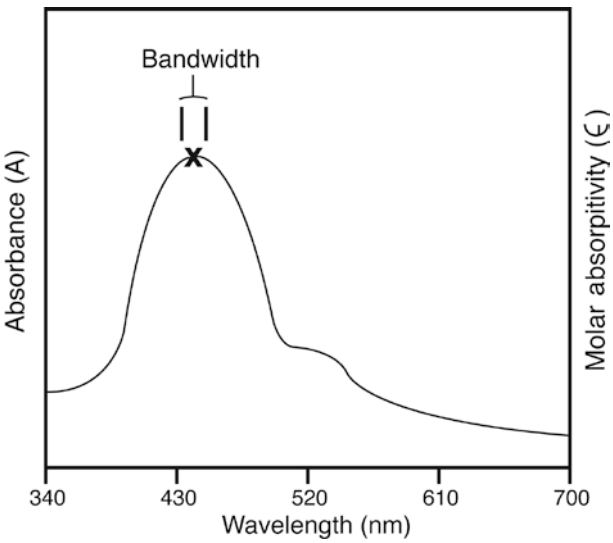
The goal of many quantitative measurements is to determine the concentration of an analyte with optimum precision and accuracy, in a minimal amount of time, and at minimal cost. To accomplish this, it is essential that the analyst consider potential errors associated with each step in a particular assay. Potential sources of error for spectroscopic assays include inappropriate sample preparation techniques, inappropriate controls, instrumental noise, and errors associated with inappropriate conditions for absorbance measurements (such as extreme absorbance/transmittance readings).

Sample preparation schemes for absorbance measurements vary considerably. In the simplest case, the analyte-containing solution may be measured directly following homogenization and clarification. Except for special cases, homogenization is required prior to any analysis to ensure a representative sample. Clarification of samples is essential prior to taking

absorbance readings in order to avoid the apparent absorption due to scattering of light by turbid solutions. The **reference solution** for samples in this simplest case will be the sample solvent, the solvent being water or an aqueous buffer in many cases. In more complex situations, the analyte to be quantified may need to be chemically modified prior to making absorbance measurements. In these cases, the analyte that does not absorb radiation in an appropriate spectral range is specifically modified, resulting in a species with absorption characteristics compatible with a given spectrophotometric measurement. Specific reactions such as these are used in many colorimetric assays that are based on the absorption of radiation in the Vis range. The reference solution for these assays is prepared by treating the sample solvent in a manner identical with that of the sample. The reference solution therefore will help to correct for any absorbance due to the modifying reagents themselves and not the modified analyte.

A **sample-holding cell** or **cuvette** should be chosen after the general spectral region to be used in a spectrophotometric measurement has been determined. Sample-holding cells vary in composition and dimensions. The sample-holding cell should be composed of a material that does not absorb radiation in the spectral region being used. Cells meeting this requirement for measurements in the **UV range** may be composed of **quartz or fused silica**. For the **Vis range** cells made of **silicate glass** are appropriate, and inexpensive **plastic** cells also are available for some applications. The dimensions of the cell will be important with respect to the amount of solution required for a measurement and with regard to the pathlength term used in Beer's law. A typical absorption cell is 1 cm^2 and approximately 4.5 cm long. The pathlength for this traditional cell is 1 cm, and the minimum volume of solution needed for standard absorption measurements is approximately 1.5 mL. Absorption cells with pathlengths ranging from 1 to 100 mm are commercially available. Narrow cells, approximately 4 mm in width, with optical pathlengths of 1 cm, are also available. These narrow cells are convenient for absorbance measurements when limiting amounts of solution are available, e.g., less than 1 mL.

In many cases an analyst must **choose an appropriate wavelength** at which to make absorbance measurements. If possible, it is best to choose the wavelength at which the analyte demonstrates maximum absorbance and where the absorbance does not change rapidly with changes in wavelength (Fig. 7.3). This position usually corresponds to the apex of the highest absorption peak. Taking measurements at this apex has two advantages: (1) maximum sensitivity, defined as the absorbance change per unit change in analyte concentration, and (2) greater adherence to Beer's law since the spectral region making up the radiation beam is composed of wavelengths with relatively small differences in their molar absorptivities for the analyte being measured (Fig. 7.3). The latter point is important in that the radiation beam used in the analysis will be composed of a small continuous band of wavelengths centered about the wavelength indicated on the instrument's wavelength selector.



7.3 figure

Hypothetical absorption spectrum between 340 and 700 nm. The effective bandwidth of the radiation used in obtaining the spectrum is assumed to be approximately 20 nm. Note that at the point indicated there is essentially no change in molar absorptivity over this wavelength range

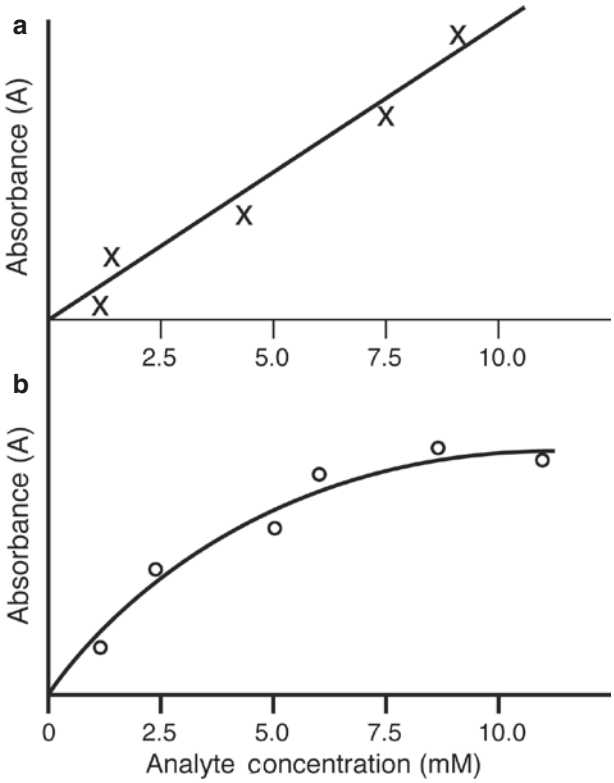
The actual **absorbance measurement** is made by first calibrating the instrument for 0% and then 100% transmittance. The 0% transmittance adjustment is made while the photodetector is screened from the incident radiation by means of an occluding shutter, mimicking infinite absorption. This adjustment sets the base level current or "dark current" to the appropriate level, such that the readout indicates zero. The 100% transmittance adjustment then is made with the occluding shutter open and an appropriate reference cell/solution in the light path. The reference cell itself should be equivalent to the cell that contains the sample (i.e., a "matched" set of cells is used). In many cases, the same cell is used for both the sample and reference solutions. The reference cell generally is filled with solvent, that often being distilled/deionized water for aqueous systems. The 100% T adjustment effectively sets $T=1$ for the reference cell, which is equivalent to defining P_0 in Eq. 7.1 as equivalent to the radiant power of the beam exiting the reference cell. The 0% T and 100% T settings should be confirmed as necessary throughout the assay. The sample cell that contains analyte then is measured without changing the adjustments. The adjustments made with the reference cell will effectively set the instrument to give a sample readout in terms of Eq. 7.6. The readout for the sample

solution will be between 0 and 100% *T*. Most modern spectrophotometers allow the analyst to make readout measurements in either absorbance units or as percent transmittance. It is generally most convenient to make readings in absorbance units since, under optimum conditions, absorbance is directly proportional to concentration. When making measurements with an instrument that employs an analog swinging needle type of readout, it may be preferable to use the linear percent transmittance scale and then calculate the corresponding absorbance using Eq. 7.3. This is particularly true for measurements in which the percent transmittance is less than 20.

7.2.4 Calibration Curves

It is generally advisable to use calibration curves for quantitative measurements. Empirical assays that require the use of a calibration curve are common in food analyses. The calibration curve is used to establish the relationship between analyte concentration and absorbance. This relationship is established experimentally through the analysis of a series of samples of known analyte concentration. The standard solutions are best prepared with the same reagents and at the same time as the unknown. The concentration range covered by the standard solutions must include that expected for the unknown. Typical calibration curves are depicted in Fig. 7.4. **Linear calibration curves** are expected for those systems that obey Beer's law. **Nonlinear calibration curves** are used for some assays, but linear relationships generally are preferred due to the ease of processing the data. Nonlinear calibration curves may be due to concentration-dependent changes in the chemistry of the system or to limitations inherent in the instruments used for the assay. The nonlinear calibration curve in Fig. 7.4b reflects the fact that the **calibration sensitivity**, defined as change in absorbance per unit change in analyte concentration, is not constant. For the case depicted in Fig. 7.4b, the assay's concentration-dependent decrease in sensitivity obviously begins to limit its usefulness at analyte concentrations above 10 m*M*.

In many cases truly representative calibration standards cannot be prepared due to the complexity of the unknown sample. This scenario must be assumed when insufficient information is available on the extent of interfering compounds in the unknown. **Interfering compounds** include those that absorb radiation in the same spectral region as the analyte, those that influence the absorbance of the analyte, and those compounds that react with modifying reagents that are supposedly specific for the analyte. This means that calibration curves are potentially in error if the unknown and the standards differ with respect to pH, ionic strength, viscosity, types of impurities, and the like. In these cases, it is advisable to calibrate the assay system by



7.4 figure

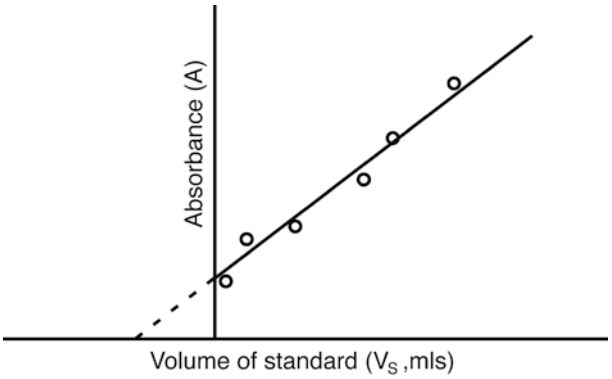
Linear (**a**) and nonlinear (**b**) calibration curves typically encountered in quantitative absorption spectroscopy

using a **standard addition protocol**. One such protocol goes as follows: to a series of flasks, add a constant volume of the unknown (V_u) for which you are trying to determine the analyte concentration (C_u). Next, to each individual flask, add a known volume (V_s) of a standard analyte solution of concentration C_s, such that each flask receives a unique volume of standard. The resulting series of flasks will contain identical volumes of the unknown and different volumes of the standard solution. Next, dilute all flasks to the same total volume, V_t. Each of the flasks is then assayed, with each flask treated identically. If Beer's law is obeyed, then the measured absorbance of each flask will be proportional to the total analyte concentration as defined in Eq. 7.8.

$$A = k\left[V_s C_s + V_u C_u\right) / \left(V_t\right)\right] \tag{7.8}$$

where:

V_s = volume of standard
V_u = volume of unknown
V_t = total volume
C_s = concentration of standard
C_u = concentration of unknown
k = proportionality constant (pathlength x absorptivity)



7.5 figure

Calibration curve for the determination of the analyte concentration in an unknown using a standard addition protocol. *A* absorbance, *Vs* volume of standard analyte solution; as discussed in text

The results from the assays are then plotted with the volume of standard added to each flask (V_s) as the independent variable and the resulting absorbance (A) as the dependent variable (Fig. 7.5). Assuming Beer's law, the line describing the relationship will be as in Eq. 7.9, in which all terms other than V_s and A are constants. Taking the ratio of the slope of the plotted line (Eq. 7.10) to the line's intercept (Eq. 7.11) and rearranging gives Eq. 7.12, from which the concentration of the unknown, C_u, can be calculated since C_s and V_u are experimentally defined constants:

$$A = kC_sV_s / V_T + V_uC_uk / V_t \tag{7.9}$$

$$\text{Slope} = kC_s / V_t \tag{7.10}$$

$$\text{Intercept} = V_uC_uk / V_t \tag{7.11}$$

$$C_u = (\text{measured intercept} / \text{measured slope})(C_s / V_u) \tag{7.12}$$

where:

V_s, V_u, V_t, C_s, C_u, and k = as in Eq. 7.8

7.2.5 Effect of Indiscriminant Instrumental Error on the Precision of Absorption Measurements

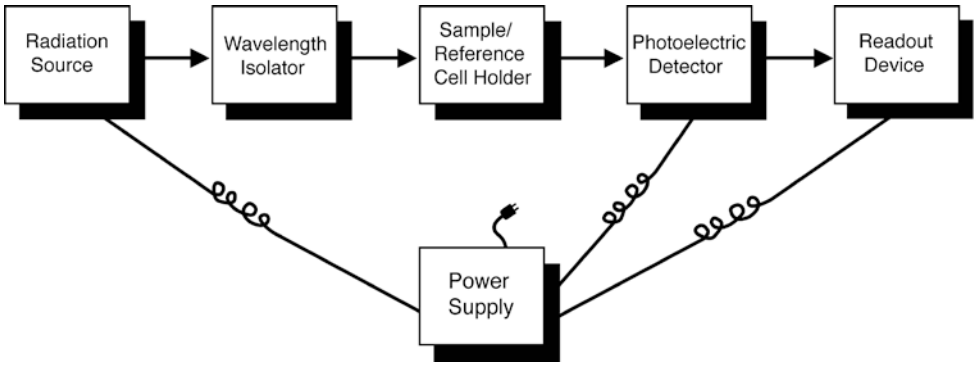
All spectrophotometric assays will have some level of **indiscriminant error** associated with the absorbance/transmittance measurement itself. Indiscriminant error of this type often is referred to as **instrument noise**. It is important that the assay be designed such that this source of error is minimized, the objective being to keep this source of error low relative to the variability associated with other aspects of the assay, such as sample preparation, subsampling, reagent handling, and so on. Indiscriminant instrumental error is observed with repeated measurements of a single homogeneous sample. The relative concentration uncertainty resulting from this error is not constant over the entire percent transmittance range (0–100 %). Measurements at intermediate transmittance values tend to have lower relative errors, thus greater relative precision, than measurements made at either very high or very low transmittance. **Relative concentration uncertainty** or relative error may be defined as S_c/C, where S_c is sample standard deviation and C is measured concentration. Relative concentration uncertainties of from 0.5 % to 1.5 % are to be expected for absorbance/transmittance measurements taken in the optimal range. The optimal range for absorbance measurements on simple, less expensive spectrophotometers is from approximately 0.2–0.8 absorbance units, or 15–65 % transmittance. On more sophisticated instruments, the range for optimum absorbance readings may be extended up to 1.5 or greater. To be safe, it is prudent to always make absorbance readings under conditions at which the absorbance of the analyte solution is less than 1.0. If there is an anticipated need to make measurements at absorbance readings greater than 1.0, then the relative precision of the spectrophotometer should be established experimentally by repetitive measurements of appropriate samples. Absorbance readings outside the optimal range of the instrument may be used, but the analyst must be prepared to account for the higher relative error associated with these extreme readings. When absorbance readings approach the limits of the instrumentation, then relatively large differences in analyte concentrations may not be detected.

7.2.6 Instrumentation

There are many variations of spectrophotometers available for UV-Vis spectrophotometry. Some instruments are designed for operation in only the visible range, while others encompass both the UV and Vis ranges. Instruments may differ with respect to design, quality of components, and versatility. A basic spectrophotometer is composed of five essential components: the **light source**, the **monochromator**, the **sample/reference holder**, the **radiation detector**, and a **readout device**. A power supply is required for instrument operation. A schematic depicting component interrelationships is shown in Fig. 7.6.

7.2.6.1 *Light Source*

Light sources used in spectrophotometers must continuously emit a strong band of radiation encompassing the entire wavelength range for which the

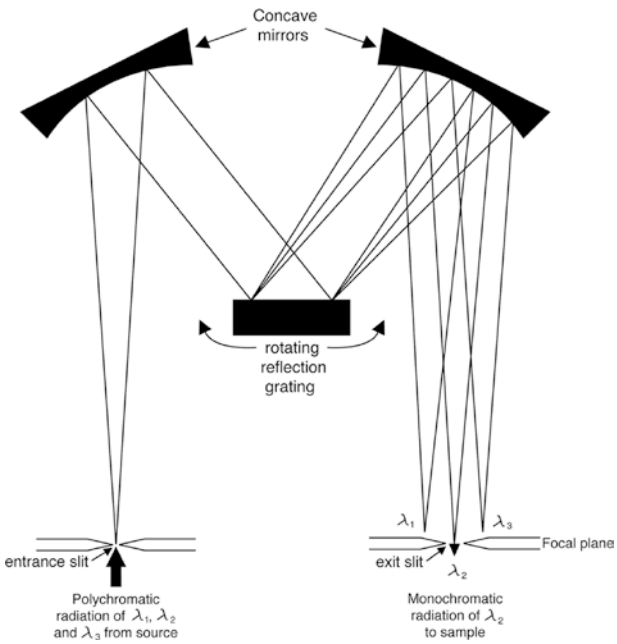


7.6 figure Arrangement of components in a simple single-beam, UV-Vis absorption spectrophotometer

instrument is designed. The power of the emitted radiation must be sufficient for adequate detector response, and it should not vary sharply with changes in wavelength or drift significantly over the experimental time scale. The most common radiation source for Vis spectrophotometers is the **tungsten filament lamp**. These lamps emit adequate radiation covering the wavelength region from 350 to 2,500 nm. Consequently, tungsten filament lamps also are employed in near-infrared spectroscopy. The most common radiation sources for measurements in the UV range are **deuterium electrical-discharge lamps**. These sources provide a continuous radiation spectrum from approximately 160 nm through 375 nm. These lamps employ quartz windows and should be used in conjunction with quartz sample holders, since glass significantly absorbs radiation below 350 nm.

7.2.6.2 *Monochromator*

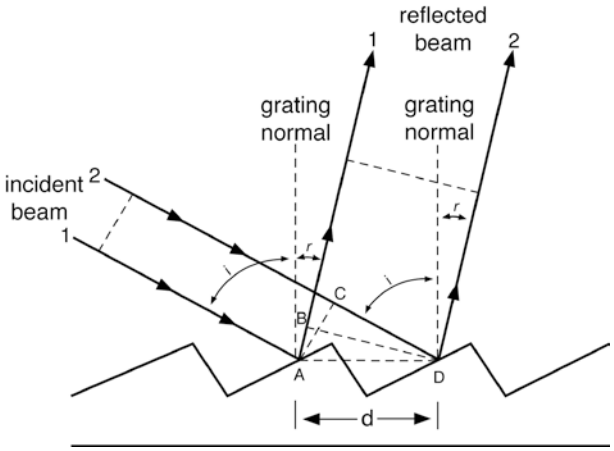
The component that functions to isolate the specific, narrow, continuous group of wavelengths to be used in the spectroscopic assay is the **monochromator**. The monochromator is so named because light of a single wavelength is termed **monochromatic**. Theoretically, **polychromatic radiation** from the source enters the monochromator and is dispersed according to wavelength, and **monochromatic radiation** of a selected wavelength exits the monochromator. In practice, light exiting the monochromator is not of a single wavelength, but rather it consists of a narrow continuous band of wavelengths. A representative monochromator is depicted in Fig. 7.7. As illustrated, a typical monochromator is composed of **entrance** and **exit slits**, **concave mirror(s)**, and a **dispersing element** (the grating in this particular example). Polychromatic light enters the monochromator through the entrance slit and is then culminated by a concave mirror. The culminated polychromatic radiation is then dispersed, dispersion being the physical separation in space of radiation of different wavelengths. The radiation of different wavelengths is then reflected from a concave



7.7 figure Schematic of a monochromator employing a reflection grating as the dispersing element. The concave mirrors serve to culminate the radiation into a beam of parallel rays

mirror that focuses the different wavelengths of light sequentially along the focal plane. The radiation that aligns with the exit slit in the focal plane is emitted from the monochromator. The radiation emanating from the monochromator will consist of a narrow range of wavelengths presumably centered around the wavelength specified on the wavelength selection control of the instrument.

The size of the wavelength range passing out of the exit slit of the monochromator is termed the **bandwidth** of the emitted radiation. Many spectrophotometers allow the analyst to adjust the size of the monochromator exit slit (and entrance slit) and, consequently, the bandwidth of the emitted radiation.



7.8 figure

Schematic illustrating the property of diffraction from a reflection grating. Each reflected point source of radiation is separated by a distance *d*

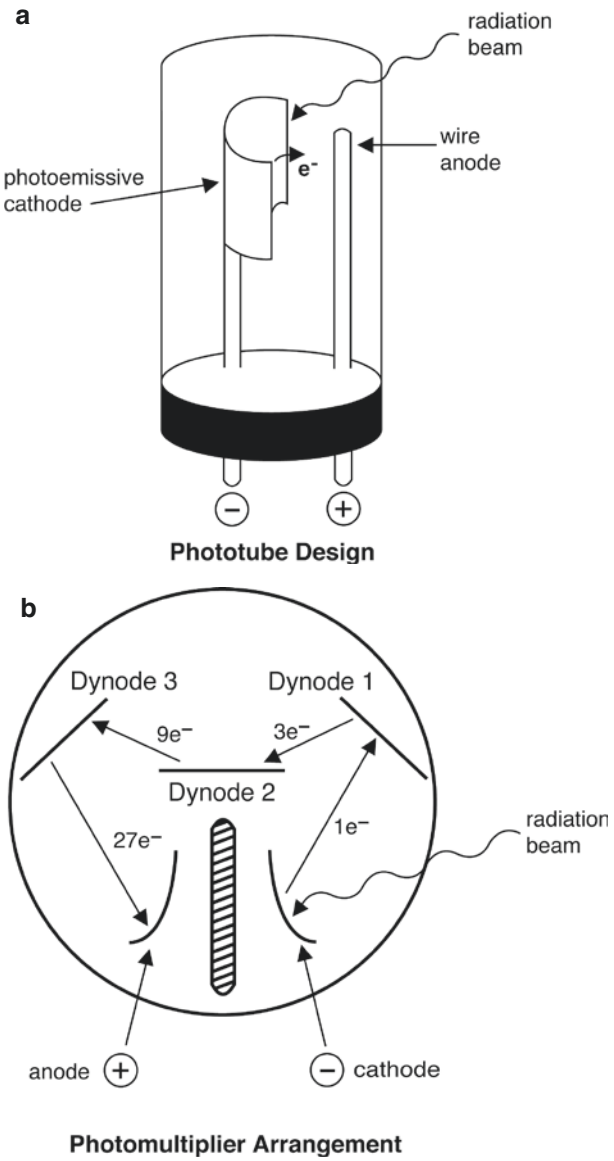
Decreasing the exit slit width will decrease the associated bandwidth and the radiant power of the emitted beam. Conversely, further opening of the exit slit will result in a beam of greater radiant power but one that has a larger bandwidth. In some cases where resolution is critical, such as some qualitative work, the narrower slit width may be advised. However, in most quantitative work, a relatively open slit may be used since adsorption peaks in the UV-Vis range generally are broad relative to spectral bandwidths. Also, the signal-to-noise ratio associated with transmittance measurements is improved due to the higher radiant power of the measured beam.

The effective bandwidth of a monochromator is determined not only by the slit width but also by the quality of its dispersing element. The dispersing element functions to spread out the radiation according to wavelength. **Reflection gratings**, as depicted in Fig. 7.8, are the most commonly used dispersing elements in modern spectrophotometers. Gratings sometimes are referred to as **diffraction gratings** because the separation of component wavelengths is dependent on the different wavelengths being diffracted at different angles relative to the grating normal. A reflection grating incorporates a reflective surface in which a series of closely spaced grooves has been etched, typically between 1,200 and 1,400 grooves per millimeter. The grooves themselves serve to break up the reflective surface such that each point of reflection behaves as an independent point source of radiation.

Referring to Fig. 7.8, lines 1 and 2 represent rays of parallel monochromatic radiation that are in phase and that strike the grating surface at an angle *i* to the normal. Maximum constructive interference of this radiation is depicted as occurring at an angle *r* to the normal. At all other angles, the two rays will partially or completely cancel each other. Radiation of a different wavelength would show maximum constructive interference at a different angle to the normal. The wavelength dependence of the diffraction angle can be rationalized by considering the relative distance the photons of rays 1 and 2 travel and assuming that maximum constructive interference occurs when the waves associated with the photons are completely in phase. Referring to Fig. 7.8, prior to reflection, photon 2 travels a distance CD greater than photon 1. After reflection, photon 1 travels a distance AB greater than photon 2. Hence, the waves associated with photons 1 and 2 will remain in phase after reflection only if the net difference in the distance traveled is an integral multiple of their wavelength. Note that for a different angle *r* the distance AB would change and, consequently, the net distance CD–AB would be an integral multiple of a different wavelength. The net result is that the component wavelengths are each diffracted at their own unique angles *r*.

7.2.6.3 *Detector*

In a spectroscopic measurement, the light transmitted through the reference or sample cell is quantified by means of a **detector**. The detector is designed to produce an electric signal when it is struck by photons. An ideal detector would give a signal directly proportional to the radiant power of the beam striking it, it would have a high signal-to-noise ratio, and it would have a relatively constant response to light of different wavelengths, such that it was applicable to a wide range of the radiation spectrum. There are several types and designs of radiation detectors currently in use. The most commonly encountered detectors are the **phototube**, the **photomultiplier tube**, and **photodiode detectors**. All of these detectors function by converting the energy associated with incoming photons into electrical current. The **phototube** consists of a semicylindrical cathode covered with a photoemissive surface and a wire anode, the electrodes being housed under vacuum in a transparent tube (Fig. 7.9a). When photons strike the photoemissive surface of the cathode, there is an emission of electrons; the freed electrons are collected at the anode. The net result of this process is that a measurable current is created. The number of electrons emitted from the cathode and the subsequent current through the system are directly proportional to the number of photons, or radiant power of the beam, impinging on the photoemissive surface. The **photomultiplier tube** is of similar design. However, in the photomultiplier tube, there is an amplification of the number of electrons collected at the anode per photon striking the photoemissive surface of the cathode (Fig. 7.9b). The electrons originally emitted from the cathode surface are attracted to a dynode with a relative positive charge. At the dynode, the electrons strike the surface, causing the emission of several



7.9 figure

Schematic diagram of a typical phototube design (**a**) and the cathode-dynode-anode arrangement of a representative photomultiplier tube (**b**)

more electrons per original electron, resulting in an amplification of the signal. Signal amplification continues in this manner, as photomultiplier tubes generally contain a series of such dynodes, with electron amplification occurring at each dynode. The cascade continues until the electrons emitted from the final dynode are collected at the anode of the photomultiplier tube. The final gain may be as many as 10^6–10^9 electrons collected per photon.

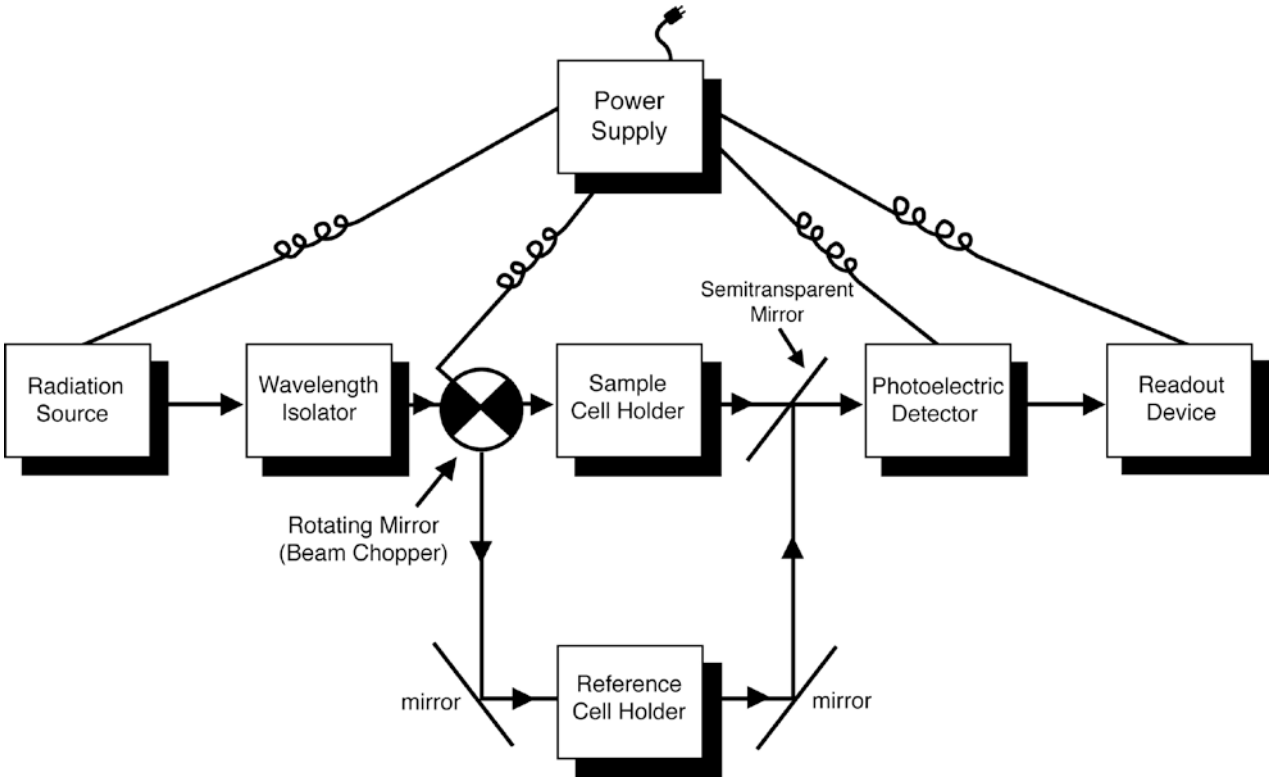
Photodiode detectors are now common in UV-Vis spectrophotometers. These are solid-state devices in which the light-induced electrical signal is a result of photons exciting electrons in the semiconductor materials from which they are fabricated, most commonly silicon. Spectrophotometers using photodiode detectors may contain a single diode detector or a linear array of diodes (diode array spectrophotometers). If a single photodiode detector is used, then the arrangement of components is generally as depicted in Fig. 7.6. If an array of photodiode detectors is used, then the light originating from the source typically passes into the sample prior to it being dispersed. The light transmitted through the sample is subsequently dispersed onto the diode array, with each diode measuring a narrow band of the resulting spectrum. This design allows one to simultaneously measure multiple wavelengths, allowing nearly instantaneous collection of an entire absorption spectrum. Diode-based detectors are generally reported to be more sensitive than phototubes but less sensitive than photomultiplier tubes.

7.2.6.4 *Readout Device*

The signal from the detector generally is amplified and then displayed in a usable form to the analyst. The final form in which the signal is displayed will depend on the complexity of the system. In the simplest case, the analog signal from the detector is displayed on an **analog meter** through the position of a needle on a meter face calibrated in percent transmission or absorbance. Analog readouts are adequate for most routine analytical purposes; however, analog meters are somewhat more difficult to read, and, hence, the resulting data are expected to have somewhat lower precision than that obtained on a digital readout (assuming the digital readout is given to enough places). **Digital readouts** express the signal as numbers on the face of a meter. In these cases, there is an obvious requirement for signal processing between the analog output of the detector and the final digital display. In virtually all cases, the signal processor is capable of presenting the final readout in terms of either absorbance or transmittance. Many of the newer instruments include microprocessors capable of more extensive data manipulations on the digitized signal. For example, the readouts of some spectrophotometers may be in concentration units, provided the instrument has been correctly calibrated with appropriate reference standards.

7.2.7 Instrument Design

The optical systems of spectrophotometers fall into one of two general categories: they are either single-beam or double-beam instruments. In a **single-beam instrument**, the radiant beam follows only one path, that going from the source through the sample to the detector (Fig. 7.6). When using a single-beam

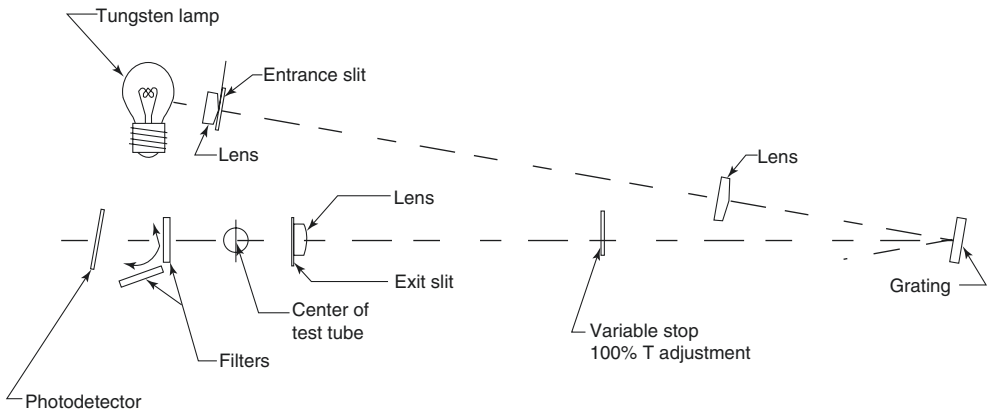


7.10 figure

Arrangement of components in a representative double-beam UV-Vis absorption spectrophotometer. The incident beam is alternatively passed through the sample and reference cells by means of a rotating beam chopper

instrument, the analyst generally measures the transmittance of a sample after first establishing 100 % T, or P_0, with a reference sample or blank. The blank and the sample are read sequentially since there is but a single light path going through a single cell-holding compartment. In a **double-beam instrument**, the beam is split such that one-half of the beam goes through one cell-holding compartment and the other half of the beam passes through a second. The schematic of Fig. 7.10 illustrates a double-beam optical system in which the beam is split in time between the sample and reference cell. In this design, the beam is alternately passed through the sample and reference cells by means of a rotating sector mirror with alternating reflective and transparent sectors. The double-beam design allows the analyst to simultaneously measure and compare the relative absorbance of a sample and a reference cell. The advantage of this design is that it will compensate for deviations or drifts in the radiant output of the source since the sample and reference cells are compared many times per second. The disadvantage of the double-beam design is that the radiant power of the incident beam is diminished because the beam is split. The lower energy throughput of the double-beam design is generally associated with inferior signal-to-noise ratios. Computerized single-beam spectrophotometers now are available that claim to have the benefits of both the single- and double-beam designs. Their manufacturers report that previously troublesome source and detector drift and noise problems have been stabilized such that simultaneous reading of the reference and sample cell is not necessary. With these instruments, the reference and sample cells are read sequentially, and the data are stored, then processed, by the associated computer.

The Spectronic® 20 is a classic example of a simple single-beam visible spectrophotometer (Fig. 7.11). The white light emitted from the source passes into the monochromator via its entrance slit; the light is then dispersed into a spectrum by a diffraction grating, and a portion of the resulting spectrum then leaves the monochromator via the exit slit. The radiation emitted from the monochromator passes through a sample compartment and strikes the silicon photodiode detector, resulting in an electrical signal proportional to the intensity of impinging light. The lenses depicted in Fig. 7.11 function in series to focus the light image on the focal plane that contains the exit slit. To change the portion of the spectrum exiting the monochromator, one rotates the reflecting grating by means of the



7.11 figure Optical system for the Spectronic® 20 spectrophotometer (Courtesy of Thermo Spectronic, Rochester, NY, Thermo Electron Spectroscopy)

wavelength cam. A shutter automatically blocks light from exiting the monochromator when no sample/reference cell is in the instrument; the zero percent *T* adjustment is made under these conditions. The light control occluder is used to adjust the radiant power of the beam exiting the monochromator. The occluder consists of an opaque strip with a V-shaped opening that can be physically moved in or out of the beam path. The occluder is used to make the 100 % *T* adjustment when an appropriate reference cell is in the instrument.

7.2.8 Characteristics of UV-Vis Absorbing Species

The absorbance of UV-Vis radiation is associated with electronic excitations within atoms and molecules. Commonly encountered analytical methods based on UV-Vis spectroscopy do not use UV radiation below 200 nm. Hence, the excitations of interest in traditional UV-Vis spectroscopy are the result of unsaturation and/or the presence of nonbonded electrons in the absorbing molecules. The UV-Vis absorption characteristics of several functional groups common to food constituents are tabulated in Table 7.2. The presented wavelengths of maximum absorbance and the associated molar absorption coefficients are only approximate since the environment to which the functional group is exposed, including neighboring constituents and solvents, will have an influence on the electronic properties of the functional group.

The type of information contained in Table 7.2 will likely be useful in determining the feasibility of UV-Vis spectroscopy for specific applications. For example, it is helpful to know the absorption characteristics of carboxyl groups if one is considering the feasibility of using UV-Vis absorption spectroscopy as a detection method to monitor non-derivatized organic acids eluting from liquid chromatography columns. With respect to this particular organic acids question, the table indicates that organic acids are likely to absorb radiation in the range accessible to most UV-Vis detectors (>200 nm). However, the table also indicates that sensitivity of such a detection method is likely to be limited due to the low molar absorption coefficient of carboxyl groups at such wavelengths. This explains why high-performance liquid chromatography methods for organic acid quantification sometimes make use of UV-Vis-detectors tuned to ~210 nm (e.g., Resource Material 3) and why there are research efforts aimed at developing derivatization methods to enhance the sensitivity of UV-Vis-based methods for the quantification of organic acids (Resource Material 11).

The data of Table 7.2 also illustrate the effect of conjugation on electronic transitions. Increased conjugation leads to absorption maxima at longer wavelengths due to the associated decrease in the electronic energy spacing within a conjugated system (i.e., lower energy difference between the ground and excited state). The aromatic compounds included in the table were chosen due to their relevance to protein quantification: benzene/phenylalanine, phenol/tyrosine, and indole/tryptophan (Chap. 18, Sect. 18.5.1). The table indicates that typical proteins will have an absorption maximum at approximately 278 nm (high molar absorption coefficient of the indole side chain of tryptophan), as well as another peak at around 220 nm. This latter peak corresponds to the amide/peptide bonds along the backbone of the protein, the rationale being deduced from the data for the simple amide included in the table (i.e., acetamide).

7.2 table Representative absorption maxima above 200 nm for select functional groups

Chromophore	*Example*	λ_{max}[a]	ε_{max}[b]	*Resource material*
Nonconjugated systems				
R-CHO	Acetaldehyde	290	17	4
R_2-CO	Acetone	279	15	4
R-COOH	Acetic acid	208	32	4
R-$CONH_2$	Acetamide	220	63	24
R-SH	Mercaptoethane	210	1,200	19
Conjugated systems				
$R_2C=CR_2$	Ethylene	<200	–	24
R-CH=CH-CH=CH-R	1,3 Butadiene	217	21,000	24
R-CH=CH-CH=CH-CH=CH-R	1,3,5 Hexatriene	258	35,000	24
11 conjugated double bonds	β-Carotene	465	125,000	13
R_2C=CH-CH=O	Acrolein (2-propenal)	210	11,500	24
		315	14	24
HOOC-COOH	Oxalic acid	250	63	24
Aromatic compounds[c]				
C_6H_6	Benzene	256	200	24
C_6H_5OH	Phenol	270	1,450	24
C_8H_7N	Indole	278	2,500	NIST database[d]

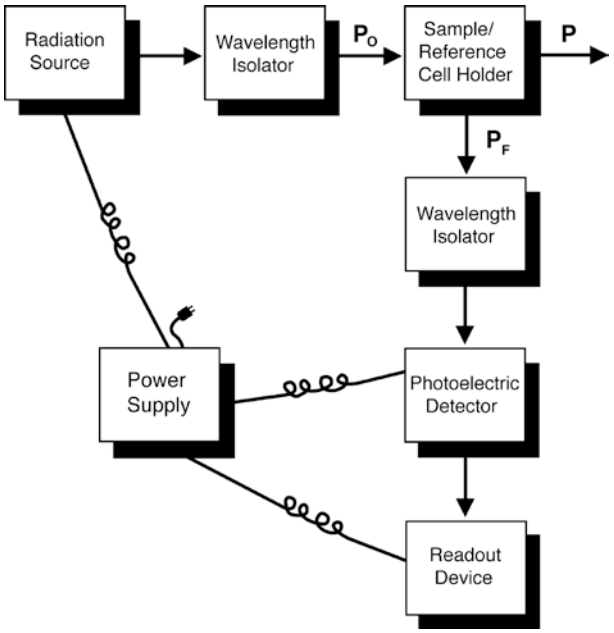
[a] λ_{max}, wavelength (in nm) of a maximum absorbance greater than 200 nm
[b] ε_{max}, molar absorption coefficient, units of $(cm)^{-1}$ $(M)^{-1}$
[c] Spectra of the aromatic compounds generally contain an absorption band(s) of higher intensity at a lower wavelength (e.g., phenol has an absorption maxima of ~210 nm with a molar absorptivity of ~6,200 $(cm)^{-1}$ $(M)^{-1}$; values from reference [1]). Only the absorption maxima corresponding to the longer wavelengths are included in the table
[d] NIST Standard Reference Database 69: *NIST Chemistry WebBook*. (The presented values were estimated from the UV-Vis spectrum for indole presented online: http://webbook.nist.gov/cgi/cbook.cgi?Name=indole&Units=SI&cUV=on. The web site contains UV-Vis data for many compounds that are of potential interest to food scientists)

7.3 FLUORESCENCE SPECTROSCOPY

Fluorescence spectroscopy is generally one to three orders of magnitude more sensitive than corresponding absorption spectroscopy. In **fluorescence spectroscopy**, the signal being measured is the electromagnetic radiation that is emitted from the analyte as it relaxes from an excited electronic energy level to its corresponding ground state. The analyte is originally activated to the higher energy level by the absorption of radiation in the UV or Vis range. The processes of activation and deactivation occur simultaneously during a fluorescence measurement. For each unique molecular system, there will be an optimum radiation wavelength for sample excitation and another, of longer wavelength, for monitoring fluorescence emission. The respective wavelengths for excitation and emission will depend on the chemistry of the system under study.

The instrumentation used in fluorescence spectroscopy is composed of essentially the same components as the corresponding instrumentation used in UV-Vis absorption spectroscopy. However, there are definite differences in the arrangement of the optical systems used for the two types of spectroscopy (compare Figs. 7.6 and 7.12). In fluorometers and spectrofluorometers, there is a need for two wavelength selectors, one for the **excitation beam** and one for the **emission beam**. In some simple fluorometers, both wavelength selectors are filters such that the excitation and emission wavelengths are fixed. In more sophisticated spectrofluorometers, the excitation and emission wavelengths are selected by means of grating monochromators. The photon detector of fluorescence instrumentation is generally arranged such that the emitted radiation that strikes the detector is traveling at an angle of 90° relative to the axis of the excitation beam. This detector placement minimizes signal interference due to transmitted source radiation and radiation scattered from the sample.

The **radiant power** of the fluorescence beam (P_F) emitted from a fluorescent sample is proportional to the change in the radiant power of the source beam as it passes through the sample cell (Eq. 7.13). Expressing this another way, the radiant power of the fluorescence



7.12 figure

Schematic diagram depicting the arrangement of the source, excitation and emission wavelength selectors, sample cell, photoelectric detector, and readout device for a representative fluorometer or spectrofluorometer

beam will be proportional to the number of photons absorbed by the sample:

$$P_F = \varphi(P_0 - P) \tag{7.13}$$

where:

PF = radiant power of beam emitted from fluorescent cell
φ = constant of proportionality
P_0 *and* P = as in Eq. 7.1

The constant of proportionality used in Eq. 7.13 is termed the **quantum efficiency** (φ), which is specific for any given system. The quantum efficiency equals the ratio of the total number of photons emitted to the total number of photons absorbed. Combining Eqs. 7.3 and 7.5 allows one to define P in terms of the analyte concentration and P_o, as given in Eq. 7.14:

$$P = P_0 10^{-\varepsilon bc} \tag{7.14}$$

where:

P_0 and P = as in Eq. 7.1
ε, b, and c = as in Eq. 7.5

Substitution of Eq. 7.14 into Eq. 7.13 gives an expression that relates the radiant power of the fluorescent beam to the analyte concentration and P_0, as shown in Eq. 7.15. At low analyte concentrations, εbc <0.01, Eq. 7.15 may be reduced to the expression of Eq. 7.16 (see Resource Material 20 for more on this). Further grouping of terms leads to the expression of Eq. 7.17, where k incorporates all terms other than P_0 and c:

$$P_F = \varphi P_0\left(1 - 10^{-\varepsilon bc}\right) \tag{7.15}$$

$$P_F = \varphi P_0\, 2.303\, \varepsilon bc \tag{7.16}$$

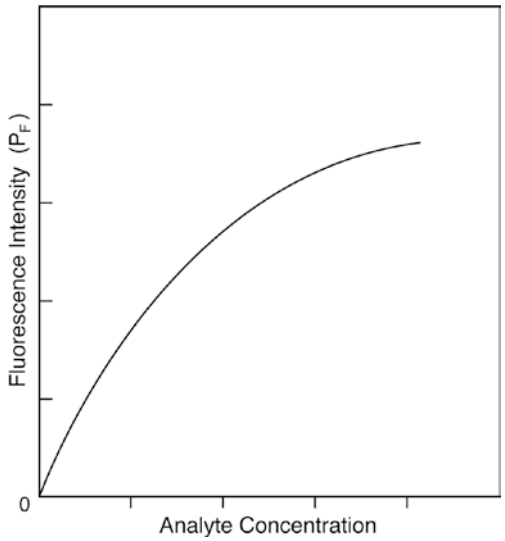
$$P_F = kP_0c \tag{7.17}$$

where:

k = constant of proportionality
P_F = as in Eq. 7.13
c = as in Eq. 7.5

Equation 7.17 is particularly useful because it emphasizes two important points that are valid for the conditions assumed when deriving the equation, particularly the assumption that analyte concentrations are kept relatively low. First, the fluorescent signal will be directly proportional to the analyte concentration, assuming other parameters are kept constant. This is very useful because a linear relationship between signal and analyte concentration simplifies data processing and assay troubleshooting. Second, the sensitivity of a fluorescent assay is proportional to P_o, the power of the incident beam, the implication being that the sensitivity of a fluorescent assay may be modified by adjusting the source output.

Equations 7.16 and 7.17 will eventually break down if analyte concentrations are increased to relatively high values. Therefore, the **linear concentration range** for each assay should be determined experimentally. A representative calibration curve for a fluorescence assay is presented in Fig. 7.13. The nonlinear portion of the curve at relatively high analyte concentrations results from decreases in the fluorescence yield per unit concentration. The fluorescence yield for any given sample also is dependent on its environment. Temperature, solvent, impurities, and pH may influence this parameter. Consequently, it is imperative that these environmental parameters be accounted for in the experimental design of fluorescence assays. This may be particularly important in the preparation of appropriate reference standards for quantitative work.



7.13 figure

Relationship between the solution concentration of a fluorescent analyte and that solution's fluorescence intensity. Note that there is a linear relationship at relatively low analyte concentrations that eventually goes nonlinear as the analyte concentration increases

7.4 SUMMARY

UV and Vis absorption and fluorescence spectroscopy are used widely in food analysis. (See Chap. 8, Table 8.2, for a comparison of these types of spectroscopy, including their applications. The table also allows for comparison with other types of spectroscopy.) These techniques may be used for either qualitative or quantitative measurements. Qualitative measurements are based on the premise that each analyte has a unique set of energy spacings that will dictate its absorption/emission spectrum. Hence, qualitative assays generally are based on the analysis of the absorption or emission spectrum of the analyte. In contrast, quantitative assays most often are based on measuring the absorbance or fluorescence of the analyte solution at one wavelength. Quantitative absorption assays are based on the premise that the absorbance of the test solution will be a function of the solution's analyte concentration.

Under optimum conditions, there is a direct linear relationship between a solution's absorbance and its analyte concentration. The equation describing this linear relationship is known as Beer's law. The applicability of Beer's law to any given assay always should be verified experimentally by means of a calibration curve. The calibration curve should be established at the same time and under the same conditions that are used to measure the test solution. The analyte concentration of the test solution then should be estimated from the established calibration curve.

Molecular fluorescence methods are based on the measurement of radiation emitted from excited analyte molecules as they relax to lower energy levels. The analytes are raised to the excited state as a result of photon absorption. The processes of photon absorption and fluorescence emission occur simultaneously during the assay. Quantitative fluorescence assays are generally one to three orders of magnitude more sensitive than corresponding absorption assays. Like absorption assays, under optimal conditions there will be a direct linear relationship between the fluorescence intensity and the concentration of the analyte in the unknown solution. Most molecules do not fluoresce and, hence, cannot be assayed by fluorescence methods.

Instruments used for absorption and fluorescence methods have similar components, including a radiation source, wavelength selector(s), sample-holding cell(s), radiation detector(s), and a readout device.

7.5 STUDY QUESTIONS

1. Why is it common to use absorbance values rather than transmittance values when doing quantitative UV-Vis spectroscopy?
2. For a particular assay, the plot of absorbance vs. concentration is not linear; explain the possible reasons for this.
3. What criteria should be used to choose an appropriate wavelength at which to make absorbance measurements, and why is that choice so important?
4. In a particular assay, the absorbance reading on the spectrophotometer for one sample is 2.033 and for another sample 0.032. Would you trust these values? Why or why not?
5. Explain the difference between electromagnetic radiation in the UV and Vis ranges. How does quantitative spectroscopy using the UV range differ from that using the Vis range?
6. What is actually happening inside the spectrophotometer when the analyst "sets" the wavelength for a particular assay?
7. Considering a typical spectrophotometer, what is the effect of decreasing the exit slit width of the monochromator on the light incident to the sample?
8. Describe the similarities and differences between a phototube and a photomultiplier tube. What is the advantage of one over the other?
9. Your lab has been using an old single-beam spectrophotometer that must now be replaced by a new spectrophotometer. You obtain sales literature that describes single-beam and double-beam instruments. What are the basic differences between a single-beam and a double-beam

spectrophotometer, and what are the advantages and disadvantages of each?

10. Explain the similarities and differences between UV-Vis spectroscopy and fluorescence spectroscopy with regard to instrumentation and principles involved. What is the advantage of using fluorescence spectroscopy?

7.6 PRACTICE PROBLEMS

1. A particular food coloring has a molar absorption coefficient of 3.8×10^3 cm^{-1} M^{-1} at 510 nm.
   (a) What will be the absorbance of a $2 \times 10^{-4} M$ solution in a 1-cm cuvette at 510 nm?
   (b) What will be the percent transmittance of the solution in (a)?

2. (a) You measure the percent transmittance of a solution containing chromophore X at 400 nm in a 1-cm pathlength cuvette and find it to be 50%. What is the absorbance of this solution?
   (b) What is the molar absorption coefficient of chromophore X if the concentration of X in the solution measured in question 2a is 0.5 mM?
   (c) What is the concentration range of chromophore X that can be assayed if, when using a sample cell of pathlength 1, you are required to keep the absorbance between 0.2 and 0.8?

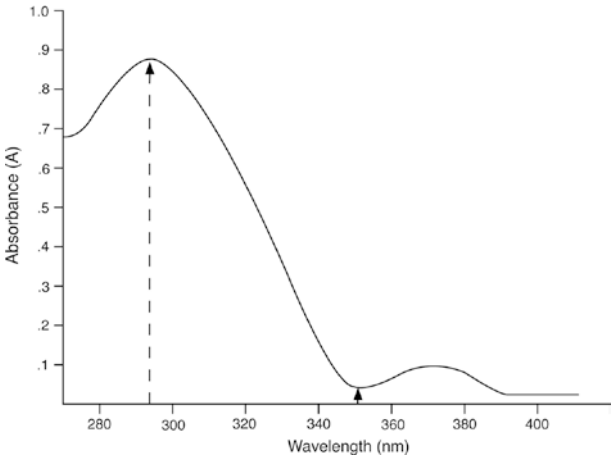
3. What is the concentration of compound Y in an unknown solution if the solution has an absorbance of 0.846 in a glass cuvette with a pathlength of 0.2 cm? The absorptivity of compound Y is 54.2 cm^{-1} (mg/mL)$^{-1}$ under the conditions used for the absorption measurement.

4. (a) What is the molar absorption coefficient of compound Z at 295 and 348 nm, given the absorption spectrum shown in Fig. 7.14 (which was obtained using a UV-Vis spectrophotometer and a 1 mM solution of compound Z in a sample cell with a pathlength of 1 cm)?
   (b) Assume you decide to make quantitative measurements of the amount of compound Z in different solutions. Based on the above spectrum, which wavelength will you use for your measurements? Give two reasons why this is the optimum wavelength.

Answers

1. (a) = 0.76, (b) = 17.4

   This problem requires a knowledge of the relationship between absorbance and transmittance and the ability to work with Beer's law.



7.14 figure — Absorption spectrum of compound Z, to be used in conjunction with problems 4a and 4b

Given: molar absorption coefficient = 3.8×10^3 cm^{-1} M^{-1}

(a) Use Beer's law: A = εbc (see Eq. 7.5 of the text)

where:

$$\varepsilon = 3.8 \times 10^3 \text{cm}^{-1} M^{-1}$$
$$b = 1 \text{cm}$$
$$c = 2 \times 10^{-4} M$$

Plugging into Beer's law gives the answer: Absorbance = 0.76

(b) Use definition of absorbance: $A = -\log T$ (see Eq. 7.3 of text)

where:

$$T = P / P_0$$

Rearranging Eq. 7.3:

$$-A = \log T$$
$$10^{-A} = T$$

$A = 0.76$ [from part (a) of question]

$$10^{-.76} = .1737 = T$$

$$\%T = 100 \times T$$

(combining Eqs. 7.1 and 7.2 of text)

Answer: $\%T = 17.4$

2. (a) = 301, (b) = 602 cm^{-1} M^{-1}, (c) = $0.33 \times 10^{-3} M$ to $1.33 \times 10^{-3} M$

   This problem again requires knowledge of the relationship between absorbance and transmittance and the manipulation of Beer's law. Care

must be taken in working with the appropriate concentration units.

(a) $T = 0.5$

$$\text{Use } A = -\log T = -\log .5 = .301$$

$$\text{Answer}: .301$$

(b) Given that the solution in part (a) is 0.5 mM (equivalent to $5 \times 10^{-4} M$)
Rearranging Beer's law: $\varepsilon = A/(bc)$

$$\varepsilon = .301 / \left[(1 \text{ cm}) \times \left(5 \times 10^{-4} M \right) \right]$$

$$\text{Answer}: \varepsilon = 602 \text{ cm}^{-1} M^{-1}$$

(c) To answer the problem, find the concentration that will give an absorbance of 0.200 (lower limit) and the concentration that will give an absorbance of 0.800 (upper limit). In both cases, use Beer's law to determine the appropriate concentrations:

where:

$$c = A / \text{eb}$$

$$\text{Lowest concentration} = 0.2/[(602 \text{ cm}^{-1} M^{-1})(1 \text{ cm})] = 3.3 \times 10^{-4} M \text{ (i.e., 0.33 m}M\text{)}$$

$$\text{Highest concentration} = 0.8/[(602 \text{ cm}^{-1} M^{-1})(1 \text{ cm})] = 1.3 \times 10^{-3} M \text{ (i.e., 1.33 m}M\text{)}$$

3. 0.078 mg/mL
This problem illustrates (1) that concentration need not be expressed in units of molarity and (2) that the pathlength of the cuvette must be considered when applying Beer's law. In the present problem the analyte concentration is given in mg/mL: thus, the absorptivity must be in analogous units:

$$\text{Apply}: c = A / \varepsilon b$$

where:

$$A = 0.846$$

$$\varepsilon = 54.2 \text{ cm}^{-1} \left(\text{mg / mL} \right)^{-1}$$

$$b = 0.2 \text{ cm}$$

$$\text{Answer}: \ 0.078 \text{ mg / mL}$$

4. (a) = 860 at 295 nm, 60 at 348 nm; (b) = 295 nm; optimum sensitivity and more likely to adhere to Beer's law.

This problem presents the common situation in which one wants to use absorbance spectroscopy for quantitative measurements but is unsure what wavelength to choose for the measurements. Furthermore, the absorptivity of the analyte at the different wavelengths of interest is unknown. A relatively simple way to obtain the necessary information is to determine the absorption spectrum of the analyte at a known concentration.

(a) The arrows on the provided spectrum indicate the points on the spectrum corresponding to 295 and 348 nm. The problem notes that the absorption spectrum was obtained using a 1 mM solution (i.e., $1 \times 10^{-3} M$ solution) of the analyte and that the pathlength of the cuvette was 1 cm. The answer to the problem is thus determined by taking the absorbance of the analyte at the two wavelengths in question and then plugging the appropriate data into Beer's law. It is somewhat difficult to get an exact absorbance reading from the presented spectrum, but we can estimate that the absorbance of the 1 mM solution is ~0.86 at 295 nm and ~0.06 at 348 nm.

$$\text{Using } \varepsilon = A / \text{bc}$$

Answer:

$$\text{At 295 nm } \varepsilon = 0.86/[(1 \text{ cm})(.001 M)] = 860 \text{ cm}^{-1} M^{-1}$$

$$\text{At 348 nm } \varepsilon = 0.06/[(1 \text{ cm})(.001 M)] = 60 \text{ cm}^{-1} M^{-1}$$

(b) In general, analysts strive to obtain maximum sensitivity for their assays, where sensitivity refers to the change in assay signal per unit change in analyte concentration (the assay signal in this case is absorbance). The absorbance values for the analyte at the different wavelengths, taken from the absorption spectrum, and/or the relative absorptivity values for the analyte at the different wavelengths, provide a good approximation of the relative sensitivity of the assay at different wavelengths (it is an approximation because we have not determined the variability/precision of the measurements at the different wavelengths). It can be seen from the given spectrum that absorbance "peaks" were at ~298 and ~370 nm. The sensitivity of the assay, relative to neighboring wavelengths, is expected to be maximum at these absorbance peaks. The peak at 295 nm is significantly higher than that at 370 nm, so the sensitivity of the assay is expected to be significantly higher at 295 nm. Thus, this would be the optimum wavelength to use for the assay. A second reason to choose 295 nm is because it appears to be in the middle of the "peak," and, thus, small changes in wavelength due to instrumental/operator limitations are not expected to appreciably change the absorptivity values. Therefore, the assay is more likely to adhere to Beer's law.

There are situations in which an analyst may choose to not use the wavelength corresponding to an overall maximum absorbance. For example, if there are known to be interfering

compounds that absorb at 295 nm, then an analyst may choose to do take absorbance measurements at 370 nm.

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