1.6 Spectrophotometry in the visible and ultraviolet regions

Absorption spectrophotometry is the measurement of the absorption, by substances, of electromagnetic radiation of definite and narrow wavelength range, essentially monochromatic.

The spectral range used in the measurements described below extends from the short wavelengths of the ultraviolet through the visible region of the spectrum. For convenience of reference, this range may be regarded as consisting of two regions, the ultraviolet (190-380 nm) and the visible (380-780 nm).

Spectrophotometry in the visible region (formerly the term colorimetry was commonly used) is the measurement of absorption of visible light, which is usually not monochromatic but restricted by the use of pigmented or interference filters.

The ultraviolet and visible spectra of a substance generally do not have a high degree of specificity. Nevertheless, they are highly suitable for quantitative assay, and for many substances they are useful as additional means of identification.

General agreement has not yet been reached on the definition of terms used in spectrophotometry. The terms in italics used in connexion with spectrophotometric tests in the *International Pharmacopoeia* are defined as follows:

Absorbance (A) - The logarithm, to the base 10, of the reciprocal of the transmittance (*T*). The term internal transmission density may be used as a synonym of absorbance; descriptive terms formerly used included optical density, absorbancy, and extinction.

Transmittance (T) - The ratio of the radiant flux transmitted by the test substance to that of the incident radiant flux. Terms formerly used include transmittancy and transmission.

Absorptivity (a) - The quotient of the absorbance (*A*) divided by the product of the concentration of the substance (*c*), expressed in g per litre, and the absorption path length (*b*) expressed in cm ($a = A/bc$). Two terms closely related to absorptivity are specific extinction and specific absorption coefficient. The term "specific extinction" ($E^{1\%}_{1\,cm}$), as generally used in pharmacopoeias, denotes the quotient of the absorbance (*A*) divided by the product of the concentration of the substance (*c*), expressed in g per 100 mL, and the absorption path length (*b*) expressed in cm; therefore $E^{1\%}_{1\,cm} = 10a$. The term "specific absorption coefficient", tentatively proposed by the Commission on Physicochemical Symbols, Terminology and Units of the International Union of Pure and Applied Chemistry (IUPAC), is defined as the quotient of absorbance (*A*) divided by the product of concentration (*c*) and the absorption path length (*l*); when the symbol a_{SI} is used for specific absorption coefficient, which in the SI should be expressed in m^2 per kg, $a_{SI} = 100a$. The term "absorptivity" is not to be confused with absorbancy index or extinction coefficient.

Molar absorptivity (ε) - The quotient of the absorbance (*A*) divided by the product of the concentration of the substance (*c*), expressed in moles per litre, and the absorption path length (*b*) in cm. It is also the product of the absorptivity (*a*) and the molecular weight of the substance. The term "molar absorption coefficient (linear)" recommended by the Commission on Physicochemical Symbols, Terminology and Units of IUPAC is defined as the quotient of the internal transmission density (absorbance) of the substance divided by the product of the concentration of the substance and the absorption path length, and according to the SI should be expressed in m^2 per mole. The terms formerly used for molar absorptivity include molar absorbancy index and molar extinction coefficient.

Absorption spectrum - The relationship of absorbance and wavelength or any functions of these, frequently represented in a graphic form.

The use of absorption spectrophotometry in the visible and ultraviolet regions for assay procedures is based on the fact that the absorptivity of a substance is usually a constant independent of the intensity of the incident radiation, the internal cell length, and the concentration, with the result that concentration may be determined photometrically.

Deviations from the above may be caused by either physical, chemical, or instrumental variables. Deviations due to instrumental error might be caused by slit-width effects, stray light, or by polychromatic radiation. Apparent failure may also result from a concentration change in solute molecules because of association between solute molecules or between solute and solvent molecules, or dissociation or ionization.

Apparatus

In essence all types of spectrophotometer are designed to permit substantially monochromatic radiant energy to be passed through the test substance in a suitable form and to allow measurement of the fraction of that energy that is transmitted. The spectrophotometer comprises an energy source, a dispersing device with slits for selecting the wavelength band, a cell or holder for the test substance, a detector of radiant energy, associated amplifiers, and measuring and recording devices. Some

instruments are manually operated, while others are equipped for automatic operation. Instruments are available for use in the visible region of the spectrum, usually 380 nm to about 700 nm, and in the visible and ultraviolet regions of the spectrum, usually 190 nm to about 700 nm.

Both double-beam and single-beam instruments are commercially available and either is suitable. Depending on the type of apparatus used, the results may be displayed on a scale, on a digital counter, or by a recorder or printer.

The apparatus should be maintained in proper working condition. The housing of the optical system should minimize any possibility of errors due to stray light; this is particularly relevant in the short-wave region of the spectrum.

Cells usually used in the spectral range discussed are 1-cm absorption cells with glass or silica windows. Other path lengths may also be used. The cells used for the test solution and the blank should be matched, and must have the same spectral transmittance when containing only the solvent. If this is not the case, an appropriate correction must be applied.

Spectrophotometer calibration

Spectrophotometers should be regularly checked for accuracy of calibrations. Where a continuous source of radiant energy is used, both the wavelength and photometric scales should be calibrated; where a spectral line source is used, only the photometric scale need be checked.

A number of sources of radiant energy have spectral lines of suitable intensity, adequately spaced throughout the spectral range selected. The exact values for the position of characteristic lines in quartz-mercury arc are 253.7, 302.25, 313.16, 334.15, 365.48, 404.66 and 435.83 nm. The wavelength scale may also be calibrated by means of suitable glass filters that have useful absorption bands through the visible and ultraviolet regions. Standard glass containing didymium (a mixture of praseodymium and neodymium) has been widely used. Glass containing holmium is considered superior. The exact values for the position of characteristic maxima in holmium glass filters are 241.5 ± 1, 287.5 ± 1, 360.9 ± 1 and 536.2 ± 3 nm. Holmium glass filters are obtainable from some national institutions and from commercial sources. The performance of an uncertified filter should be checked against one that has been properly certified. The wavelength scale may also be calibrated using holmium perchlorate TS. The exact values for the position of characteristic maxima of this solution are as follows: 241.15 nm, 278.2 nm, 361.5 nm and 536.3 nm. It should be noted that the position of characteristic maxima of holmium perchlorate solutions and holmium glass filters may differ slightly.

For the calibration of the photometric scale the tolerance generally permitted is ± 1% of the absorptivity. For checking this scale potassium dichromate TS may be used. The exact values of absorbance and specific extinction for a solution of potassium dichromate containing exactly 60.06 mg in 1000 mL of sulfuric acid (0.005 mol/l) VS at an absorption path length of 1.000 cm and the permitted tolerances for *A* are given below:

Wavelength	235 nm (minimum)	257 nm (maximum)	313 nm (minimum)	350 nm (maximum)
A	0.748	0.865	0.292	0.640
Permitted tolerance	0.740-0.756	0.856-0.874	0.289-0.295	0.634-0.646
$E^{1\%}_{1cm}$	124.54	144.02	48.62	106.56

A number of standard inorganic glass filters of known transmittance produced for checking the photometric scale are also available from some national institutions and from commercial sources but may require periodic calibration.

Operation of spectrophotometers

Detailed instructions for operating spectrophotometers are supplied by the manufacturers. To achieve significant and valid results, the operator of a spectrophotometer should be aware of its limitations and of potential sources of error and variation. The instruction manual should be followed closely on such matters as care, cleaning, and calibration of the instrument, and instructions for operation. Where double-beam recording instruments are used the cell containing solvent only is placed in the reference beam.

The cleanliness of absorption cells should receive particular attention. Usually, after treatment with an appropriate cleansing medium, the cells should be rinsed with distilled water and then with a volatile organic solvent to promote drying. Test solutions should not be left in the cells longer than necessary for carrying out the measurement. When handling the cells, special care should be taken never to touch the external surfaces through which the light beam passes. When the solvent and the test solution are transferred to the cells, care is to be taken that the liquids do not contaminate the outer surfaces.

Solvents for use in the ultraviolet region

Many solvents are suitable for tests and assays using spectrophotometry in the ultraviolet region. Water, alcohols, chloroform, lower hydrocarbons, ethers, and dilute solutions of ammonium hydroxide, sodium hydroxide, sulfuric acid and hydrochloric acid

can be used for this purpose. The solvents differ as to the lower wavelength at which the decrease in transparency prevents their use. Precautions should be taken to use solvents free from contaminants absorbing in the relevant spectral region. Specially purified solvents for spectrophotometric determinations are available commercially from several sources but need only be used when the spectral characteristics of the usual analytical grade of solvent are inadequate for a particular purpose.

The absorbance of the solvent cell and its contents should not exceed 0.4 per cm of path length when measured with reference to air at the same wavelength. The solvent in the solvent cell should be of the same batch as that used to prepare the solution and must be free from fluorescence at the wavelength of measurement. Ethanol (~750 g/l), dehydrated ethanol, methanol, and cyclohexane used as solvents should have an absorbance, measured in a 1-cm cell at 240 nm with reference to water, not exceeding 0.10.

Identification tests in the ultraviolet region

The monographs describing qualitative tests involving spectrophotometry in the ultraviolet region specify the concentration of the solution and the path length. In such tests it is more convenient to use a recording instrument. If the conditions stated are not appropriate for a particular instrument, the thickness of the solution should be varied and not the concentration.

Some identification tests involving spectrophotometry require the use of reference substances, generally an International Chemical Reference Substance (ICRS). The reference substance is then to be prepared and simultaneously measured under conditions identical for all practical purposes to those used for the test substance. Unless otherwise specified in the monograph, in making up the solution of the reference substance, a solution of about the desired concentration (i.e. within 10% of the value) should be prepared. Identical conditions for the measurement include the following: wavelength setting, slit-width adjustment, cell placement, and correction and transmittance levels.

A useful approach for identification tests in the ultraviolet region is to quote the ratio of absorbance values at two maxima. This procedure minimizes the influence of instrumental variations on the test and obviates the need for a reference substance.

Quantitative determinations in the ultraviolet region

Spectrophotometric assays usually call for a comparison of the absorbance produced by the solution of the test substance, prepared as specified in the monograph, with the absorbance of a solution of a reference substance. In such cases the spectrophotometric measurements are made first with the solution prepared from the reference substance and secondly with the solution prepared from the substance to be examined. The second measurement is carried out as quickly as possible after the first, using the same experimental conditions.

Spectrophotometric assays are usually carried out at a peak of spectral absorption for the compound concerned. The monographs give the commonly accepted wavelength for peak spectral absorption of the substance in question. It is known that different spectrophotometers may show minor variations in the apparent wavelength of this peak. Good practice demands that use be made of the peak wavelength actually found in the individual instrument, rather than the specific wavelength given in the monograph, provided the two do not differ by more than ± 0.5 nm in the 240-280 nm range, by more than ± 1 nm in the 280-320 nm range, and by more than ± 2 nm above 320 nm. If the difference is greater, recalibration of the instrument may be indicated.

The solution of the reference substance, generally an ICRS, is to be prepared and measured in the same manner as described for "Identification tests in the ultraviolet region". The calculations should be made on the basis of the exact amount weighed and, if the reference substance used has not previously been dried, on the dried or anhydrous basis. Specific instructions in individual monographs indicate the manner in which a reference substance is to be dried or treated prior to use. These instructions are to be followed unless otherwise specified in the individual test or assay, or in the labelling.

To ensure that the conditions used are appropriate the monograph may also specify the value of absorbance of a 1-cm layer of the reference substance. In this case the determination carried out against the reference substance is considered valid when the observed value of absorbance is within the range of values specified in the monograph.

For quantitative work, a manually scanning instrument is frequently used. When a recording instrument is used for that purpose special attention should be paid to proper calibration of the absorbance scale at the wavelength used.

Quantitative determinations are usually carried out at wavelengths above 235 nm. If the measurements are to be made at a wavelength in the 190-210 nm range, special precautions should be observed, for example, purging the cell compartment with nitrogen, use of solvents of special spectrophotometric quality, and using cells that are transparent in this region.

When measuring the absorbance at an absorption maximum the spectral slit-width must be small compared with the half width of the absorption band, otherwise an erroneously low absorbance will be measured. Particular care is needed for certain substances and the instrumental slit-width used should always be such that further reduction does not result in an increased absorbance reading. Problems may be encountered, owing to diffraction of the light beam, at slit-widths below 0.01 mm.

When the assays are carried out with routine frequency it is permissible to omit the use of a reference substance and use instead a suitable standard curve prepared with the respective ICRS. This may be done when, for the substance tested, the absorbance is proportional to the concentration within the range of about 75-125% of the final concentration used in the assay. Under these

circumstances, the absorbance found in the assay may be interpolated on the standard curve, and the assay result calculated therefrom. Such standard curves should be confirmed frequently, and always when a new apparatus or new lots of reagents are put into use. In the event of uncertainty or dispute, direct comparison with an ICRS must be made.

Quantitative determinations in the visible region

Spectrophotometric assays in the visible region, as in the ultraviolet region, usually call for simultaneous comparison of the absorbance produced by the assay preparation with that produced by a standard preparation containing approximately an equal quantity of a reference substance.

For spectrophotometric assays in the visible region the recommendations given under "Quantitative determinations in the ultraviolet region", including those concerning the use of standard curves, should be followed with suitable modifications, where necessary. In this region, observed wavelengths should not differ by more than 5 nm from that specified in the monograph.