Calibration and Monitoring of Spectrometers and Spectrophotometers

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Introduction

The percentage of quantitative analyses performed in the clinical laboratory that involve spectrophotometry or colorimetry was estimated in 1969 to be possibly more than 59% (1). Although this percentage may have declined somewhat in the last 10 years, most laboratories continue to rely heavily upon spectrometers (single-beam instruments) or spectrophotometers (double-beam instruments) for the majority of their analyses. Maintenance of a properly functioning spectrophotometer is an obvious prerequisite to the assurance of accurate analytical results. Moreover, the increased regulation of the clinical laboratory by governmental and professional agencies mandates that laboratory personnel periodically verify that a given spectrophotometer is functioning properly. By periodically inspecting spectrometric functions, subjective or gradual degradations in performance can be detected before they significantly affect analytical results. As a minimum, these inspections should include checks of wavelength calibration, linearity of detector response, and checks on the presence of “stray radiation.”

The procedures presented here are used regularly in our laboratory for checking spectrometric functions. Other useful checks and various instrument-affected results are also discussed.

Wavelength Accuracy

According to Beer's law \( A = abc \), absorbance, \( A \), is a function of absorptivity, \( a \), cell path length, \( b \), and concentration, \( c \). Ordinarily, the absorbance of the chromophore is measured at the wavelength of maximum absorptivity. Naturally, if the wavelength calibration of an instrument changes, the absorbance will change. The magnitude of the absorbance error due to inaccurate wavelength calibration is dependent on the relative location of the point on the absorption spectrum; that is, the absorbance error relative to the wavelength error is greater when the absorbance measurement is on the slope of the absorbance band than when the absorbance measurement is on or near the peak of the absorbance band (2).

Maintenance of wavelength calibration is especially important for analyses such as colorimetric assays of enzymes, in which a previously prepared curve of absorbance vs. concentration is used to convert absorbance readings to concentration units.

Periodic checks are necessary to ensure that the instrument's wavelength reading accurately reflects the wavelength of energy passing through the exit slit of the monochromator. Several methods are available for checking the wavelength accuracy of a spectrometer or spectrophotometer. The most accurate methods involve replacing the source lamp with a radiating energy source that has strong emission lines at well-defined wavelengths. Use of radiating energy sources are the mercury vapor lamp and deuterium or hydrogen lamps. Spectrophotometers equipped with a hydrogen or deuterium radiating-energy source have built-in sources for checking wavelength accuracy at any time (3).

Another method for checking wavelength calibration involves the use of rare-earth glass filters such as holmium oxide or didymium. Holmium oxide has strong absorption lines at approximately 241, 279, 287, 333, 361, 418, 453, 536, and 636 nm; didymium has much broader absorption bands at 573, 586, 685, 741, and 803 nm (4, 5). These filters are usually available from instrument manufacturers in a form that allows direct insertion into the sample compartment. The disadvantage of these filters is that they must be used to locate wavelengths of transmittance minima, which is intrinsically more difficult than locating wavelengths of transmittance maxima (2). When the holmium oxide filter is used in a single-beam instrument, continuous adjustments must be made to obtain a stable baseline over the entire wavelength range (6). Because of the possibility of filter deterioration, the wavelength accuracy of these filters should be checked periodically.

A third method for checking wavelength calibration involves the use of solutions. For example, under well-defined conditions, a solution of samarium oxide exhibits a characteristic spectrum with 13 sharp absorption bands (4). Disadvantages of chemical solutions are that the absorption peaks are generally broad and spectral shifts may result from contamination, aging, or preparation errors (2). A stable colored solution can be used as a secondary wavelength calibration standard to determine whether the wavelength accuracy of an instrument has changed after the wavelength accuracy has been certified by a primary wavelength calibration standard such as a mercury or deuterium lamp. One of the procedures we discuss uses such a secondary standard, in this case a solution of green food coloring, to determine whether the wavelength accuracy changes after the accuracy has been certified by use of a mercury or deuterium lamp.

Irrespective of the method of wavelength calibration, calibration at more than one wavelength is required for proper calibration of the instrument. For prism instruments, which
Wavelength Calibration Check with a Mercury Lamp (1, 3)

Principle. Because it has many emission lines between 300 and 600 nm, a quartz mercury lamp is useful for checking the wavelength calibration of spectrophotometers and spectrometers in the ultraviolet and visible regions of the spectrum. To check the wavelength calibration, substitute the mercury lamp for the usual radiant energy source(s). The wavelength reading at the point of maximum deflection should coincide with the known emission peaks of the mercury lamp.

Equipment. Pen-Ray quartz lamp and SCT-1 power supply (Ultra-Violet Products, Inc., San Gabriel, CA 91778).

Procedure.
1. Make sure the instrument is in the single-beam mode of operation.
2. To ensure stability, allow the instrument to warm up for the length of time recommended in the users' manual.
3. Make sure the radiant-energy source in the spectrometer or spectrophotometer is off. In some instruments it may be necessary to disconnect one "lead" from the radiant energy source to the power supply; in other instruments the radiant energy source may have to be removed.
4. Position the quartz mercury lamp so that its maximum energy will fall upon the entrance slit of the monochromator.
5. Rotate the wavelength selector several nanometers to either side (scan from longer to shorter wavelengths) of the wavelengths listed below and record the wavelength reading at the point of maximum deflection of the readout meter.

Interpretation. The wavelengths at which maximum deflection occurs should correspond to the mercury emission lines (313, 365, 405, 436, and 546 nm) ± 1 nm. The fact that wavelength calibration is correct at one point in the spectrum in no way assures correct calibration elsewhere. Therefore, more than one point for calibration is needed (see above).

Wavelength Calibration Check with Deuterium or Hydrogen Lamps (3, 5)

Principle. This quick and accurate check of wavelength accuracy is useful for any ultraviolet–visible spectrophotometer that (a) has a deuterium or hydrogen lamp whose radiant energy can be reflected to the entrance of the monochromator and still have the wavelength varied in the visible region of the spectrum, and (b) can be operated as a single-beam instrument. (Many double-beam instruments can be manually set in a mode of operation in which only the total energy of a single beam is recorded, not its intensity relative to a standard or blank.) Deuterium or hydrogen lamps have intense emission lines at 656 and 486 nm. The instrument is scanned a few nanometers on either side of these wavelengths.

Procedure.
1. Turn on the deuterium or hydrogen lamp and allow the instrument to warm up properly.
2. Place the source selector in the ultraviolet position.
3. Put the spectrophotometer in the single-beam configuration.
4. Set the wavelength to 656 nm.
5. Adjust the sensitivity control at a high gain (to allow use of a narrow slit).
6. Adjust the slit (for adjustable-slit instruments) to the narrowest setting that will still allow detection of meter deflection.
7. Set the wavelength to 660 nm and slowly scan manually to 650 nm (scan from longer to shorter wavelengths); note the wavelength at which maximum meter deflection occurs.
8. Set the wavelength to 486 nm.
9. Adjust the sensitivity control and slit width to as narrow a slit setting as possible that will still allow detection of meter deflection.
10. Set the wavelength to 490 nm and slowly scan manually to 480 nm (scan from longer to shorter wavelengths). Note the wavelength at which maximum deflection occurs.
11. Repeat steps 6–10 to verify the wavelengths of maximum deflection.

Interpretation. The wavelengths at which maximum deflection occurs should be 656 (±1) and 486 (±1) nm. If the wavelengths found are not within these tolerance limits the spectrophotometer is considered to be out of calibration.

Wavelength Calibration Check with Green Food Coloring

Principle. The wavelength calibration check determines whether the wavelength accuracy of the instrument has changed. This procedure is to be used after the wavelength accuracy of the spectrometer or spectrophotometer has been certified by use of a mercury or deuterium lamp. We follow this procedure at least weekly in our laboratory. A cuvette should be selected that is used only for the wavelength calibration check; this cuvette must be used on subsequent calibrations.

The absorption spectrum of the green dye solution shows maxima at 257, 410, and 630 nm. The wavelengths chosen to monitor for changes in calibration, 370 and 650 nm, occur on steep slopes of the spectrum, where a relatively small change in wavelength produces relatively large changes in absorbance.

Reagent. Dilute 0.50 mL of green food coloring (H. T. French Co., Rochester, NY 14609; obtained from a local grocery) to 2 L with water in a volumetric flask; this solution is stable for six months when stored in a brown glass bottle at room temperature. The green food coloring consists of FDC yellow No. 5 and FDC blue No. 1, 5 parts yellow to 1 part blue, 25 g/L in water and propylene glycol. Two other commercial green food colorings—Durkee (Cleveland, OH 44115) and McCormick (Baltimore, MD 21202), which exhibit identical absorption spectra, may be used.

Procedure.
1. Turn on the instrument and allow sufficient time for warm-up.
2. Measure A or %T of the wavelength calibration check solution vs. a water blank at 650 nm.
3. Measure A or %T of the check solution vs. a water blank at 370 nm.

Interpretation. The %T of the solution at 650 and 370 nm should be ±2% of the %T obtained after the wavelength accuracy of the instrument is certified by use of a mercury or deuterium lamp. At 650 nm, a change of 2% T corresponds to a change in wavelength of approximately 2 nm. For instruments measuring absorbance, the allowable limits would be the absorbance range corresponding to ±2% T of the absorbance obtained after the wavelength accuracy is certified by a mercury or deuterium lamp. Each time a new solution is prepared, the allowable limits must be recalculated from the mean %T or A obtained from at least 20 separate measurements of the new solution. If the %T or A is not within the allowable limits, the wavelength calibration of the instrument must be confirmed with the mercury or deuterium lamp.
Linearity of Detector Response

A properly functioning spectrometer or spectrophotometer must exhibit a linear relationship between the radiant energy absorbed and the instrument readout (6). Instrumental linearity is a prerequisite for spectrophotometric accuracy as well as for analytical accuracy. Several methods have been proposed for certifying that the detector response of spectrometers and spectrophotometers is linear over the range of wavelengths used (1, 6-8). Solid glass filters may be used to check instrumental linearity (6). Knowledge of the exact absorbance of the filter is not necessary, but the absorbance should be small compared to the total linear range of the instrument. When a filter is used, check linearity by comparing the change in absorbance at different positions in the linear range (6).

The most common method for certifying linearity of detector response is through the use of solutions of compounds known to follow Beer’s law. Although it has been stated that the fulfillment of Beer’s law is a necessary but not sufficient condition of spectrophotometer linearity (7), the alternative procedures suggested for checking linearity are so elaborate and tedious that the use of solutions remains the most practical procedure available in the clinical laboratory.

The Subcommittee on Spectrophotometry of the Standards Committee of the American Association for Clinical Chemistry recommended in 1969 that photometric linearity be checked by preparing dilutions of appropriate compounds (1). Compounds reported for this purpose (1, 8) include oxyhemoglobin at 415 nm, p-nitrophenol at 405 nm, cobalt ammonium sulfate at 512 nm, copper sulfate at 650 nm, and cyanmethemoglobin at 540 nm. The method presented below uses a water-soluble green dye with absorbance maxima at 257, 410, and 630 nm. The advantages of using this solution for monitoring linearity are several: one solution has absorption maxima in the opposite ends of the visible spectrum as well as in the ultraviolet region; it is safe, inexpensive, readily available, and easy to use; and its absorbance is not dependent on pH from 2.5 to 10.0 or on temperature from 4 to 56°C (8).

A nonlinear response for a plot of absorbance vs. concentration indicates either an error in dilution or an instrumental problem. Besides a faulty detector, “stray radiation” or too wide a slit width may cause a nonlinear response (1, 9). Because linearity tends to decrease as the band width increases, the slope of the absorbance vs. concentration curve may vary from instrument to instrument (1).

Linearity of Response

Principle. The linearity of detector response is verified weekly at two or three different wavelengths by serial dilutions of a dye known to absorb at 257, 410, and 630 nm.

Reagents
1. Stock green dye solution. Dilute 0.10 mL of green food coloring (H. T. French Co.) to 200 mL with water. This solution is stable for six months when stored in a brown glass bottle at room temperature.
2. Working green dye solutions. Make the following serial dilutions of the stock green dye solution (prepare freshly each day):

| Solution No. | 5.0 mL of | 5.0 mL of | % of stock concn.
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<td>3</td>
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Fig. 1. Effect of stray radiation (1% and 5%) on absorbance

Procedure
1. Turn on the instrument and allow sufficient time for warm-up.
2. Measure the transmittance or absorbance of the four working green dye solutions vs. a water blank at 257, 410, and 630 nm, using the same cuvette, starting with the blank and proceeding through the most concentrated solution.
3. Plot %T vs. % of stock dye concentration on semilog graph paper or plot absorbance vs. concentration on coordinate graph paper.

Interpretation. Plots at all three wavelengths should exhibit linear response over the entire concentration range used. The data may also be subjected to linear regression analysis and the slope and intercept recorded. In our laboratory, the slope determined each week is compared with previous checks of spectrometric functions to determine whether the value of the slope is changing with time. For each linearity check, the intercept should approximate zero. If the plots do not show linearity, the test should be repeated to exclude dilution as a source of error. If the repeated test indicates nonlinearity, take appropriate actions (such as replacement of the detector or notification of the instrument serviceman).

Stray Radiation

Stray radiation (often called “stray light”) has been defined in many ways (1, 2, 4-6, 9-13). Radiant energy that reaches the detector at wavelengths other than those indicated by the monochromator setting, and all radiant energy that reaches the detector without having passed through the sample, are considered stray radiation. Stray radiation is usually expressed as a ratio or percentage of the total radiation detected (10, 12). Considering stray radiation as a ratio, one can see that an increase in stray radiation may be due not only to an increase in the spurious radiation but also to a decrease in the primary radiation without a corresponding decrease in the spurious radiation (10). This explains why an increase in stray radiation is often observed at the extreme ends of the spectral range, where detector response or source energy is at its lowest (4, 10).

There are many sources of stray radiation other than decreased source energy intensity or decreased photodetector...
response. Some stray radiation may be due to extraneous light that reaches the detector through an improperly closed sample compartment or a leak in the detector housing; it can be detected by "scanning" the exterior of the instrument with a bright light when the instrument is being used at a wavelength in the visible range (9); unwanted radiant energy reaching the detector is detected by observation of spurious meter deflection. Masking the suspected source of the light leak with black tape will often eliminate this source of stray radiation.

Stray radiation may originate from many sources in the instrument itself, such as an improper source lamp (2). This contribution of stray radiation increases as the lamp ages (14) and is more pronounced at the lower end of the useful range (2). The effect of an aging source should be detected by a nonlinear response to the check for linearity of detector response. Misalignment of the sample cell or cuvette is still another possible source of stray radiation (15).

The chief source of stray radiation originating in the monochromator is the scattering of light by dirty or imperfect optical surfaces, or by reflection from slit edges, baffles, collimator, or any other exposed edges of components. Another source of stray radiation caused by the monochromator is second-order radiation (radiation at one-half of the chosen wavelength) passed by a grating monochromator (2).

Stray radiation causes deviation from Beer’s law, usually negative, although it is possible to obtain positive deviations if the stray radiation is absorbed (4). Figure 1 illustrates the effect of stray radiation (1 and 5%) on absorbance. Note that the nonlinearity becomes more apparent at increasing concentrations. Thus, if a nonlinear response is observed at the higher concentrations when linearity of detector response is being monitored (see check in the preceding section), the cause may be either stray radiation or a faulty detector. Significant stray radiation may also cause the appearance of false absorbance bands (1).

Methods for detecting stray radiation involve substances, either filters or solutions, for which there is little absorption over a portion of the spectrum but which are essentially opaque below an abrupt “cutoff” wavelength. Several such solutions have been used to check for stray radiation (4, 10, 12), including saturated lithium carbonate below 250 nm (4), NaBr (0.1 mol/L) below 240 nm (12), and acetone below 320 nm (12). The exact wavelength at which the cutoff occurs is a function of concentration, cell path length, and temperature (12), so the wavelengths given may vary somewhat. Many filters can detect stray radiation; Slavin (12) and Poulson (10) list some of the filters available.

If solutions or filters that transmit no radiant energy at the measurement wavelength are used, the measured transmittance is the amount of stray radiation present. Multiplication of this transmittance by 100 gives the percentage of stray radiation. Instrument malfunction is indicated by stray radiation exceeding 1% (9, 13).

Procedures taken to eliminate stray radiation include (13) verifying wavelength calibration, sealing light leaks, detecting phototube energy output, realigning instrumental components, and cleaning optical surfaces. Many of these procedures should be performed by a competent serviseman. Sometimes stray radiation can be reduced by using filters that absorb strongly at the wavelengths where the stray radiation arises but transmit well in the region where measurements are made (12).

Photometric Accuracy

When performing analyses that do not use chemical standards, absorbance accuracy is essential. An absorbance standard (6) should have constant, stable absorbance over a suitable wavelength range that is insensitive to the spectral bandwidth of the instrument and to variations in the geometry of the light beam; and it should be easy to use, readily available, and inexpensive. The National Bureau of Standards (NBS) has a set of three neutral-density glass filters, SRM 930 (6), which have known absorbances at four wavelengths for each filter. These filters are not completely stable and must be recalibrated periodically by NBS.

Several solutions have been proposed as standards for photometric accuracy certification (1, 4, 11). Potassium dichromate solutions are probably the most extensively studied and used. High-purity potassium dichromate is available from NBS. Other chemicals advocated as standards are cobalt ammonium sulfate and potassium nitrate (1). Comparing the absorbance values from these standard solutions with known absorbance values allows certification of absorbance accuracy. Standard solutions are subject to absorbance changes with time, temperature, and pH, which makes them unsuitable as long-term calibration standards for photometric accuracy (11).

Additional Factors Affecting Instrument Precision

Many other factors can affect the quality of the photometric result obtained. The instrument slit-width affects absorbance in that increasing the slit-width lowers absorbance values and increases absorbance errors (11). This source of error may be minimized by decreasing the slit-width until there is no further change in the recorded spectrum (11). Slit-widths should be as small as possible within the limits imposed by the necessity of maintaining the best signal-to-noise ratio.

The type of detector used affects the relative concentration error (11). With the older photoconductive detectors the optimum absorbance range for obtaining the most precise results was near 0.43 absorbance units, whereas with the modern photomultiplier tubes the optimum absorbance range for highest precision is broader and extends to higher absorbance, near 1.00 A (11).

Photometric accuracy is also affected by such factors as position of the sample cell and the manner in which the cell is cleaned (11).

Summary

We have delineated some of the factors affecting the performance of spectrometers and spectrophotometers in the clinical laboratory and have presented some of the methods for verifying that these instruments are functioning properly. At a minimum, every laboratory should perform periodic inspections of spectrometric functions to check wavelength calibration, linearity of detector response, and stray radiation. Only through such an inspection program can a laboratory ensure that these instruments are not contributing to inaccurate analytical results.

References


Editor’s note: The reader is reminded that Selected Methods do not bear the official imprimatur of the Association. They are methods that seem durable and generally useful, and that have been checked by several evaluators. As detailed elsewhere [Clin. Chem. 19, 1207 (1973)], these methods are offered here for criticism by the world community of users, and will be revised appropriately before being collected into a bound volume, Selected Methods of Clinical Chemistry. The last such volume was published by the Association in 1977.

No reprints of these papers will be available, because they are not regarded as necessarily being final versions.