Basic Principles of Spectrophotometry
Part II

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Spectrophotometry

• Basic Principle
  – Monochromatic light passed through absorbing solution of fixed depth (cuvette)
  – Transmitted light directed to photosensitive device
  – Radiant energy converted to electrical energy
  – Instrument adjusted using blank containing all components of unknown solution except the substance being measured

Spectrophotometry - Instrument

• Lamp
• Monochromator: prism vs diffraction grading

Spectral Bandwidth/Bandpass

• Monochromator – light obtained not truly one wavelength, a range of wavelengths
• Spectral Bandwidth (Bandpass)
  – Range of wavelengths transmitted to cuvette
  – Selected wavelength = 450 nm; bandpass = 10 nm
  – Range of wavelengths through cuvette = 445-455 nm
Spectral Bandwidth: Affects Linearity and Resolution
- Narrow bandpass is desirable to increase
  - Sensitivity, linearity, resolution

Spectral Bandwidth: Affects Linearity
- Narrow bandpass: increases linearity

Spectral Bandwidth: Affects Resolution
- Narrow bandpass: increases resolution and sensitivity

Spectrophotometry – Sources of Error
- Test sample
  - Lipemia: false increase absorbance
  - Hemolysis
    - False ↑ absorbance at specific wavelengths
    - Interfere with chemical rxn: false ↑ or ↓
    - Released intracellular components = ↑ in LD, K⁺, Mg²⁺, folate, hemoglobin
  - Icterus – amber to orange due to bilirubin pigment may interfere at specific wavelengths

Spectrophotometry – Sources of Error
- Temperature variation; temp not optimal
- pH variation
- Standards & Standardization
  - Highest purity of standards and water
  - Precise measurement
- Preparation of Solutions/Reagents
  - Precise measurement
  - Chemically pure water; highest grade
  - Mix contents well
Spectrophotometry – Sources of Error

- Cuvettes
  - Clean, dry, not scratched (use kimwipes)
  - Remove air bubbles from solution
- Wavelength selection – not optimal or calibration error
- Presence of stray light - ↓ABS & ↑%T

Spectrophotometry – Sources of Error

- Incorrect blank used – should be similar to matrix of sample
- Particulates in solution - ↓%T, centrifuge sample to clear sample of particulates
- Clerical errors
  - Most common laboratory error
  - Always recheck work

Spectrophotometry – Maintenance & QC

- Purpose of PM & QC
  - Verify instrument performance
  - Maintain optimal instrument performance
  - Ensure precision and accuracy of measurement

Spectrophotometry – Maintenance & QC

- Preventive Maintenance (PM) consists of:
  - Checking specific instrument parameters
    - Wavelength Accuracy - Stray Light Detection
    - Photometric Linearity - Baseline Stability
    - Photometric Accuracy - Temperature Calibration
  - Cleaning waste container
  - Cleaning surface and interior components
  - Changing tubing, light source, etc.

Spectrophotometry – Maintenance & QC

- When is PM performed?
  - Routine (daily)
  - Monthly
  - Bi-annually
- After PM performed – run controls (QC) to verify instrument is working properly

Wavelength Accuracy

- Verifies the radiant energy emitted from monochromator exit slit is the same wavelength selected on the selector dial
- Checked whenever a new lamp is used and then routinely thereafter
- Methods
  - Nickel sulfate solution measurements at 460 and 550 nm
  - Didymium filter peaks 585 nm
  - Cobalt chloride solution peaks 510 nm
  - Potassium dichromate solution peaks 350, 375 & 450 nm
  - Transmission stds from NIST: didymium (45%T @ 610 nm)
- Correction: realign exciter lamp w/wavelength selector
Linearity of Detector Response (Photometric Linearity)

- Verifies a linear relationship exists between radiant energy absorbed by solution and the instrument readout (ABS vs conc is linear)

- Method
  - Varying concentrations of soln known to follow Beer’s law
  - ABS vs. Conc is plotted, straight line = linear response

- Correction
  - Recheck dilutions
  - Check for stray light (most common), failing photocell, incorrect slit width

Photometric Accuracy

- Checks for changes in bandpass & amount of light energy falling on the photocell

- Method – nickel sulfate (510 nm)

- Correction
  - Realign exciter lamp
  - Clean exciter lamp or photocell window
  - Correct faulty slit width
  - Replace damaged diffraction grating

Stray Light

- Checks for stray light striking detector which will falsely increase %T and falsely decrease ABS

- Method
  - Observed at extreme ends of spectrum, where detector response or source energy at lowest
  - Nickel sulfate (400nm & 700nm)
  - Sodium nitrite solution (<0.1%T @ 355 nm)

- Correction
  - Clean or replace exciter lamp
  - Find source of spurious light reflection: check mirrors, prisms, gratings, dust on optical surfaces

Baseline Stability

- Baseline stability
  - Detects excess baseline drift

- Method:
  - Observe ABS (or %T) at a chosen wavelength
  - Look for >2% change within 1 minute

- Correction: change failing exciter lamp

Temperature

- Ensures cuvette temp is accurate & stable
  - Especially important w/enzyme assays

- Method
  - Thermometer or thermistor: NIST certified
  - Temperature sensitive dye: cresol-red/Tris buffer soln