Chapter 1

Quantitative Instrumental Measurements

1.1. INTRODUCTION

This chapter introduces the basic principles underlying many common methods of signal *transduction*. This term is used to describe the conversion of one type of energy to another. Generally, analytical specialists use the term transducer to describe the conversion of a concentration (or mass) into a useful electronic signal, which is ultimately almost always a voltage. This voltage is related to the concentration (or mass) of the *analyte*, or species of interest, in the original sample. The species that can be measured by one or more of these methods is not always the analyte itself; for example, if the analyte is an enzyme or other catalytic species, the depletion of reactants or accumulation of products is assessed based on their own unique properties.

Transduction can be accomplished in many different ways, and the choice of the best method depends on which of many possible physical properties are exhibited by the measured species. In this chapter, we consider the three main types of transduction that are widely used in instrumental methods in bioanalytical chemistry. The conversion of light into current is performed by photodiodes or photomultipliers, and this current is then electronically converted into a voltage that is proportional to the intensity of the light. Electrochemical and surface plasmon resonance transducers convert chemical energy into a measured voltage or into a current that is subsequently converted to a voltage. Scintillation counters, used in many radiochemical methods, first convert beta-particle radioactivity to light, and the light is detected using photodiodes or photomultipliers. Thermal transducers, used for calorimetry, convert heat into current (and then voltage).

Considerations for the choice of a transduction method include the uniqueness of the various measurable properties of the measured species, since it is often present in a complicated sample *matrix*. The matrix is the surrounding environment, and includes all other components present in the sample. Matrix components can interfere with measurements in direct or indirect ways: a matrix component may exhibit a

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similar physical property to the analyte, and interfere with analyte measurement; also, a matrix component may interact with the analyte, changing the nature of its physical property and/or the magnitude of its resulting signal.

This chapter is intended as an introduction and brief review of common transduction methods used in bioanalytical chemistry. More detailed descriptions of applications and instrumental variations will be found within specific chapters of this book, where more specialized adaptations are described for specific assay methods.

The reader is referred to two excellent analytical chemistry textbooks for greater depth of coverage of most of the basic descriptions given in this chapter, as well as two excellent review articles for more information on thermal measurement methods, listed at the end of the chapter.

1.2. OPTICAL MEASUREMENTS

The majority of quantitative optical methods make use of light that is either absorbed or emitted in the ultraviolet and visible regions of the electromagnetic spectrum. These regions formally correspond to wavelengths of 1.0×10^{-8} to 7.8×10^{-7} m, and are more commonly expressed in nm units (10 to 780 nm). The far UV region, also called the vacuum UV region, is generally not analytically useful, but the near UV and the visible regions are widely used.

The colours that surround us result mainly from wavelength-selective visible light absorption by molecules present in the items that we see. However, differences between species, and between individuals within a species, cause the wavelength range of visible light, and the colours within this range, to be perceived differently. Common examples are bumblebees, that have blue-shifted visible ranges, and hummingbirds, that have red-shifted ranges. For this reason, standard wavelength ranges have been defined for the different colours of the visible spectrum. For example, blue light is defined as the 440–470 nm range, and if blue light is absorbed, its complementary colour, orange, is observed. Similarly, if green light (500–520 nm) is absorbed, purple is the observed colour. Many compounds absorb light at multiple wavelengths, and it is the combination of complementary colours that we observe.

The relationship between wavelength, frequency and energy of light is shown below:

$$E = h\upsilon = hc/\lambda, \tag{1.1}$$

where E is the energy of the light, h is Planck's constant (6.626×10^{-34} J·s), υ is the frequency of the light (s^{-1}), λ is the wavelength of the light (m), and c is the speed of light (2.998×10^{8} m/s in a vacuum, and this number is divided by the refractive index n for any other medium). This relationship connects the two key concepts that light is both a particle (a photon with energy E) and a wave, with frequency υ and wavelength λ.

In the visible and near UV regions of the spectrum, molecules absorb and emit light as their electronic configurations change. For example, electrons convert between paired and unpaired states, or between bonding and non-/antibonding orbitals. These conversions are accompanied by energy gains or losses as the molecule absorbs or emits a photon. Depending on molecular structure, as well as many other factors including solvent, pH and temperature, fixed electronic energy levels exist, and only photons of particular energies (wavelengths) can be absorbed or emitted. Associated with each electronic energy level are vibrational and rotational energy levels, which are separated by much smaller energy differences. Isolated vibrational or rotational transitions can be made to occur using infrared or microwave radiation, which have much lower energy. But the electronic transitions that occur in the UV-visible region are accompanied by vibrational and rotational transitions, and this means that a range of wavelengths can be absorbed by molecules, shown in Eq. 1.2:

$$\Delta E_{\mathrm{T}} = \Delta E_{\mathrm{Elec}} + \Delta E_{\mathrm{Vib}} + \Delta E_{\mathrm{Rot}}, \tag{1.2}$$

where, for a given electronic transition, the total energy ΔE_{T} of the photons absorbed is the sum of the energy required for the electronic transition itself, ΔE_{Elec}, which is fixed, plus the energy changes associated with multiple possible vibrational and rotational transitions, ΔE_{Vib} and ΔE_{Rot}. This means that, for any given electronic transition, molecules absorb or emit a fairly wide range of wavelengths, centered on a wavelength of maximal absorption or emission. For molecules absorbing or emitting light in the near UV and visible regions, the range of wavelengths can be as large as 100 nm for a given electronic transition, because of these accompanying vibrational and rotational transitions.

1.2.1. UV-Visible Absorbance

A simple spectrophotometer, an instrument for measuring absorbance, consists of a light source, a monochromator (or filter), a sample compartment and a light detector, all of which are enclosed to prevent interference from ambient light. These components are shown as a block diagram in Figure 1.1. Typically, the light source is a tungsten filament lamp (for the visible region) and/or a deuterium lamp (for the UV region); both of these sources emit continuous radiation over a wide range of wavelengths. Wavelength selection can be accomplished using filters, for repetitive fixed-wavelength measurements, or a monochromator containing a diffraction grating or prism, that allows adjustment of wavelength as well as wavelength scanning. The quality of the filter or monochromator determines the width of the wavelength range in the light beam that exits the device and is directed into the sample. Analyte solutions are contained in a cuvette (or cell) made of a material that is transparent to the wavelength(s) of interest, such as quartz, glass or polystyrene. Light detection may be accomplished using a photomultiplier tube, a photodiode, or a photodiode array (in which the spatial distribution of light of different wavelengths allows nearly instantaneous acquisition of a complete spectrum).

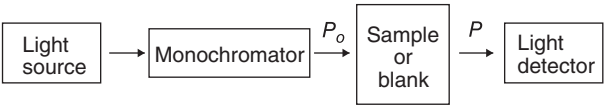

Figure 1.1. Block diagram of a simple UV-Vis absorption spectrophotometer.

Many variations of this simple design have been introduced for specialized applications. For example, dedicated instruments may employ an inexpensive light-emitting diode as the light source, a combination of absorption and interference filters for wavelength selection, or a flow cell in which a solution continuously flows past the light beam. In all cases, the instruments are designed to measure the absorption of light by an analyte.

The intensity, or power, of the incident monochromatic beam of light is given the symbol P_O, and the light intensity that exits the sample compartment has the symbol P. Commonly, P_O is measured using a reagent blank solution, i.e. a solution containing all of the components of the sample solution except the analyte. Transmittance, T, is the ratio of these values (P/P_O), and may be expressed as this simple ratio, with a value between zero and one, or as a percentage that ranges from zero to one hundred.

As the concentration of the analyte in the sample cell is increased, the transmittance decreases, but the dependence of transmittance on concentration is not linear. For quantitative purposes, transmittance values are converted to absorbance (A) values as follows:

$$A = -\log T = \log(P_o/P) \tag{1.3}$$

Absorbance increases linearly as analyte concentration is increased. It also increases linearly with the distance through which the light beam travels in the sample; this is called the path length and is given the symbol b. The Beer-Lambert Law (Eq. 1.4), also called Beer's Law, is the most important relationship in quantitative spectrophotometry.

$$A = \varepsilon bc \tag{1.4}$$

In this relationship, absorbance A depends linearly on analyte concentration c with two proportionality constants: b, the path length, and ε, the molar absorptivity of the analyte. Absorbance is unitless, and so the units of ε are generally $M^{-1}cm^{-1}$, when analyte concentration is in molar units and path length is expressed in cm.

Absorbance is additive. If there is more than one absorbing species present in a solution, the total absorbance at a given wavelength is the sum of the absorbances of the individual species at that wavelength. This property is analytically useful for the quantitation of multiple absorbing species, if the molar absorptivities are known at multiple wavelengths. The concentrations of two components, for example, can be determined by measuring absorbances at two wavelengths, at which the molar absorptivities of the two components are known.

Absorbance spectra, plots of absorbance against wavelength, are used to determine the best wavelengths for analyte quantitation. A maximum, or peak, in this plot indicates the wavelength at which ε has its highest value, and at this wavelength, the slope of the calibration curve of A vs. c (the sensitivity) will be maximal. There may be several peaks in an absorption spectrum; the choice of the best wavelength to use for quantitation depends on both the molar absorptivities at the peaks and the likelihood of interfering species absorbing light at the chosen wavelength.

Most bioanalytical methods focus on analytes that are present in aqueous solutions, and most of these analytes have charge states that are pH dependent. Absorption spectra can change quite dramatically as pH is varied, because the electronic energy levels change with the protonation state of the molecule. Molecules containing carboxylic acid and amine groups, for example, exhibit pH-dependent absorption spectra. Some of the most dramatic examples of this effect are found in small molecules that are used as visible indicators in pH titrations. Control of the pH of the analyte and standard solutions is thus critical.

Molar absorptivity also depends on temperature, ionic strength and solvent. Its value, if needed, is commonly determined from the slope of a plot of absorbance against concentration, using a calibrated cuvette with a known path length.

1.2.2. Turbidimetry (Light-Scattering)

These methods employ the same instruments used for absorbance spectrophotometry. They are applied to turbid solutions, meaning that the solutions appear cloudy due to suspensions of particulate matter. The particles can be cells or precipitates formed by reactions. An apparent absorbance value is measured, for which the scattering, rather than the absorption of light is the cause. Often the term "optical density" is used rather than absorbance. Light scattering tends to increase as the wavelength decreases toward the UV region of the spectrum, but absorbance, rather than scattering, also increases at lower wavelengths. Turbidity is typically measured at a wavelength near 540 nm, where absorbance is rare. Optical density depends on particle size, and, for a constant particle size and low concentrations, is linearly related to particle concentration.

Cells in suspension are often quantitated by turbidimetry. Calibration is required for each species, by correlation with plate counts, or colony-forming units. A second area of wide application involves latex particles that are surface-modified to react with a soluble analyte. This causes clumping of the particles, or a growth in average particle size, allowing the quantitation of the soluble analyte by turbidimetry. These latex agglutination tests are discussed in more detail in Chapter 6.

1.2.3. Fluorescence

The instruments used for fluorescence spectroscopy are more complicated than those used for absorbance measurements. They use a very high-intensity light source, such as a xenon arc lamp, and they require two monochromators, as shown in Figure 1.2.

Incident monochromatic radiation is used to excite the analyte, i.e. to change its energy from the ground to an excited electronic state. This incident light is directional, meaning that a beam of light is directed through the sample. During the lifetime of the excited state, which is typically 0.01–100 μs, the molecule rotates and can lose vibrational and rotational energy through radiationless deactivation. Emission of a photon then allows the molecule to return to the ground electronic state. Emitted photons are generally of lower energy (longer wavelength) because of radiationless deactivation. The difference between the excitation and the emission wavelengths is called the Stokes shift. For instruments that are used only for fluorimetry, P is not measured, and this light is simply absorbed by the flat black internal walls of the instrument housing. In some instruments, P_o is measured as a reference signal (in case of fluctuations in lamp intensity) using a beam splitter and a reference light detector; with this configuration, the sample emission is related to P_o, and this provides improved precision.

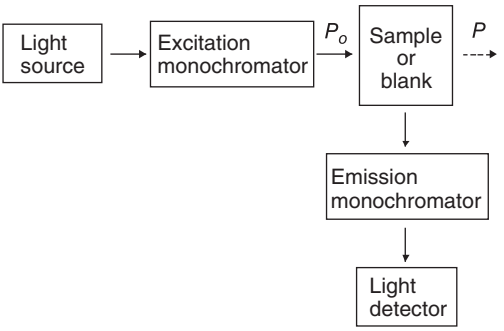

Figure 1.2. Block diagram of a simple spectrofluorimeter.

From a quantitative perspective, the finite lifetime of the excited state is very significant. Excited state molecules rotate randomly, and when photons are emitted by fluorescent molecules in solution, they are emitted in all directions. This means that emitted light can be collected at right angles to the incident excitation beam, and this is the common detection geometry for stand-alone fluorescence instruments. This collection geometry minimizes background light that reaches the detector. This geometry has important analytical implications: for fluorescent molecules, quantitation by fluorescence can be as much as 1000-fold more sensitive than quantitation by absorbance.

The appropriate fixed wavelengths for the two monochromators are chosen by sequentially scanning wavelengths with each device to obtain excitation and emission spectra. An excitation spectrum is obtained by monitoring the emission at a fixed wavelength, and scanning the excitation wavelengths; it is a plot of emission intensity against excitation wavelength. A maximum in the excitation spectrum indicates the appropriate fixed wavelength to use in order to obtain the

emission spectrum. The emission spectrum is acquired by fixing the excitation wavelength and scanning the emission wavelength; it is a plot of emission intensity against emission wavelength. For a new analyte, an absorbance spectrum may be acquired initially, to aid in the selection of an appropriate excitation wavelength.

Once these wavelengths have been determined, the monochromators are fixed at these values, and a calibration is performed. At low concentrations of fluorescing species, fluorescence intensity is directly proportional to concentration. The proportionality constant (or the slope of the calibration curve) depends on many factors including molar absorptivity and quantum yield of the analyte, as well as a number of instrumental parameters that determine how much light is collected and how monochromatic it is when it is detected.

A molecule's quantum yield is the ratio of the number of molecules that emit a photon to the number of molecules that are in the excited state. The value lies between zero and one, with one representing the most fluorescence. Structurally rigid, aromatic molecules and functional groups (and some amino acid side chains) tend to exhibit strong fluorescence. Factors such as solvent, pH, dissolved oxygen concentration and temperature also affect fluorescence.

Self-absorption is a phenomenon that occurs when the concentration of the fluorescent species is high. Under these conditions, emitted light is absorbed by nearby fluorescent molecules that are not already in an excited state. Calibration curves that extend to high concentrations generally exhibit a linear region, at low concentration, followed by a maximum and then a continuous decrease in fluorescence intensity as concentration increases.

Quenching of fluorescence can occur when a different molecular species is able to absorb photons that are emitted by a fluorescent molecule. This concept has been used in a number of important bioanalytical methods and devices that are described in later chapters.

Specialized fluorescence measurement methods include time-resolved fluorescence and fluorescence polarization, and are largely employed when fluorescent labels are used in homogenous immunoassays. In these methods, the persistence of fluorescence with time, or the polarization of the light emitted after excitation by polarized light, is measured. For certain useful labels, these properties change significantly when the fluorescently-labelled molecule interacts with its binding partner.

1.2.4. Chemiluminescence and Bioluminescence

Excited-state molecules can be generated as products of chemical or biochemical reactions. When the excited-state reaction products return to their ground states, photons are emitted at characteristic wavelengths. A well-known example of bioluminescence involves the firefly: the enzyme luciferase generates an excited-state product, and when the product molecules emit photons, the fireflies glow in the dark.

The instrumentation needed for these measurements is very simple: a housing painted flat black on the inside, containing a sample compartment, light collection optics and a light detector such as a photomultiplier tube or photodiode. Filters and monochromators are possible, but are generally not needed for bioanalytical measurements.

The signal obtained from chemi- and bioluminescence measurements is fundamentally different from that obtained from fluorescence measurements. Instead of the continuous absorption and emission of photons, as occurs in fluorescence, photons are produced stoichiometrically, as products of the (bio)chemical reaction. For this reason, a limited number of photons are produced, and can be detected, with these methods, and when the reaction has proceeded to completion, no further photons will be produced.

Instruments used for these measurements generally allow the integration of the signal from the light detector. When signal integration is performed for a fixed time interval following the start of the reaction, these integrated signals are proportional to the reaction rate and thus to the concentrations of reactants.

1.3. ELECTROCHEMICAL MEASUREMENTS

This family of measurement methods is based on reduction-oxidation (redox) reactions in which one or more electrons are stoichiometrically transferred from a reduced species to an oxidized species. These types of reactions are widespread in biological and biochemical systems, and are the basis of respiration in living organisms. Aerobic organisms, after a long series of enzyme-catalyzed redox reactions, ultimately transfer electrons to molecular oxygen, reducing it to water.

An overall, or net, electron transfer reaction can also be made to occur through an external circuit that separates two containers, each containing one of the two reactants. An electrode, made of an inert material such as platinum or carbon, is placed in each container, and if these two electrodes are connected with, e.g. a copper wire, electrons will flow from the container containing the reduced form to the one containing the oxidized reactant. The circuit is completed by a salt bridge that allows the movement of ions from the container with the oxidized reactant to that with the reduced reactant, to compensate for electron flow and maintain electroneutrality. Thus, electrons flow through the external circuit, while ions flow between the solutions. The transduction event, the conversion of ion current into electronic current, occurs at the surfaces of the two electrodes, where electrons are either accepted from or donated to the reacting species in the solutions.

In each of these two containers, a half-reaction (either an oxidation or a reduction) occurs. When the two reactions are balanced for the number of electrons transferred, and then summed, a net, or overall cell reaction is obtained. The standard reduction potential, E^o, and the formal reduction potential, $E^{o\prime}$, of each of the two half reactions are thermodynamic properties that indicate the propensity of the reactant to either accept or donate electrons. The standard reduction potential value applies to unit activities of the reacting species, and is not widely used for biological

systems. The formal reduction potential, however, is widely used, and values are based on specified conditions of pH, temperature and ionic strength. These values are widely available in tables.

For analytical purposes, we are generally interested in only one of the two half-reactions, but the second half-reaction is needed to complete the circuit to allow the measurement to occur. The second half-reaction can be made to occur under constant conditions in a reference half-cell, also called a reference electrode, that is contained in, typically, a small glass tube that is separated from the analyte solution by a porous glass frit, which acts as a salt bridge, allowing the movement of ions. Various geometric options exist for the manufacture of electrochemical cells, including screen-printing of both electrodes/half cells onto flat plastic, disposable substrate materials that are very useful for the measurement of blood glucose and lactate levels when a drop of blood is placed onto the surface, connecting the two electrodes via the mainly aqueous liquid.

The key equation for most electrochemical measurements is the Nernst Equation (Eq. 1.5), which, when constants are evaluated for 25 °C and collected into one term, simplifies to Eq. 1.6:

$$E = E^{o\prime} - (RT/nF)ln([R]/[O]) - E_{ref} \tag{1.5}$$

$$E = \text{Constant} - (0.05916)\log([R]/[O]), \tag{1.6}$$

where R is the Rydberg constant, T is absolute temperature, F is Faraday's constant and n is the number of electrons involved in the reduction of the oxidized species, O, to the reduced form, R:

$$O + n\,e^{-} = R. \tag{1.7}$$

Strictly, activities, rather than concentrations, exist in the Nernst Equation; the activity coefficients are also collected into the *Constant* term of Eq. 1.6. In these expressions, E (on the left side) represents the net cell potential, in volts, and this is the driving force for the overall reaction. It is important to note that E is related to the logarithm of the concentration ratio.

Typically, in biochemical or organic chemical redox reactions, protons, as well as electrons are involved in half-reactions:

$$O + m\,H^{+} + n\,e^{-} = R, \tag{1.8}$$

where m is the stoichiometric factor for protons in the reaction, and often, m has the same value as n. Because of this pH dependence, and because standard conditions involve unit activity of reactants (i.e. 1 M acid, or a pH of zero), formal reduction potentials, $E^{o\prime}$, are used, and are determined by measurements made when $[O] = [R]$, under specified conditions of pH, temperature and ionic strength. Buffers are used to

maintain constant pH, and thus pH-related terms in the Nernst Equation are collected into the value of the *Constant* shown in Eq. 1.6.

For analytical purposes, it is generally unnecessary to know the exact values of $E^{o\prime}$ or E_{ref}, as long as they are constant, since calibration curves are generated.

1.3.1. Potentiometry

Potentiometric methods employ a high-impedance voltmeter to measure the value of E while preventing the overall cell reaction from occurring to any significant extent.

The most widely familiar example of potentiometric measurement involves the combination pH electrode. In this device, two reference half cells are incorporated into a single probe, using concentric tubes. The half cells are connected by a porous glass frit, exposed to the external solution. The key element in the pH electrode is a glass membrane that separates one of the reference half-cells from the external (analyte or calibration) solution. The glass membrane allows the selective transport of protons (hydronium ions) and thus incorporates an ion-selective connection between the two reference electrodes. The ionic or solution connection is thus from one reference electrode, through the glass membrane, through the analyte solution, through the glass frit (salt bridge) into the second reference electrode compartment. If the two reference electrodes are identical, any difference in proton concentration (pH) on the two sides of the glass membrane generates a measurable, nonzero potential difference that is calibrated to the pH of standard solutions.

The glass membrane of a pH electrode can be covered with a gas-only-permeable membrane, trapping a thin layer of weakly buffered solution between the two membranes. With this arrangement, dissolved gas present in the analyte solution can cross into the thin layer. Due to their acid-base equilibria, both ammonia (NH_3/NH_4^+) and carbon dioxide (CO_2/HCO_3^-) can be quantitated using these devices, in which the measurement of the pH of the thin layer of solution is calibrated against the concentration of the dissolved gas in the analyte solution.

Various other ion-selective membrane materials have been developed to allow potentiometric measurements of anions and cations. With all ion-selective electrodes, calibration curves are constructed as plots of measured potential against the logarithm of the ion concentration. At 25 °C, the slopes of these calibration curves are equal to 0.05916/z V/decade, where z is the charge on the ion (e.g. ±1, ±2). Thus, a Ca^{2+} ion-selective electrode will produce a slope of +0.02958 V/decade, while a Cl^- electrode will yield a slope of −0.05916 V/decade.

1.3.2. Amperometry

Amperometric measurements involve the application of a potential E to the cell (Eq. 1.6), to control the $[R]/[O]$ ratio at the surface of the working, or indicator electrode (where the reaction of interest occurs). If this dictated ratio is different from the

ratio existing in the solution, current flows, and current is the measured parameter. The reactions only occur at the electrode surfaces, and not in the bulk of the solution, so transport of the analyte to the working electrode surface is a key factor in determining the magnitude of the current that is measured. Flowing or stirred solutions can be used to enhance mass transport.

Electrodes used for amperometry are good conductors, such as platinum, gold or different forms of carbon. They may be thinly coated with a blocking agent to prevent the adsorption of reactants, products or other constituents present in the analyte solution that would cause fouling of the electrodes and thus decreased currents.

When the applied potential is sufficiently extreme, all analyte that reaches the working electrode surface reacts. Under these conditions, the measured current is directly proportional to analyte concentration. Current may also be integrated for a defined period of time, to provide the total charge consumed during this integration time. Charge is also directly proportional to analyte concentration.

One widely-used application of amperometry involves the Clark oxygen electrode. This small, self-contained device contains two electrodes, one of which converts molecular oxygen to hydrogen peroxide. An inner solution is separated from the external analyte solution using a gas-permeable membrane, allowing only dissolved gases to cross into the inner solution. The applied potential is controlled to allow mass-transport-limited reduction of oxygen, and the resulting current is proportional to dissolved oxygen concentration in the external solution. Many enzymatic reactions that consume oxygen (e.g. the oxidases) have been studied with the aid of the Clark electrode.

1.3.3. Impedimetry

In bioanalytical chemistry, impedance measurements can be used to monitor changes in the ionic strength, or conductivity of a solution as a result of a (bio)chemical reaction. In weakly-buffered solutions, reactions that produce or consume ions can be monitored by impedimetry.

With these methods, a small, alternating (sinusoidal) voltage is applied across two electrodes that are present in the solution. This alternating waveform is centered on zero volts, and its small peak-to-peak magnitude ($\leq$50 mV) and high frequency (kHz) prevent redox reactions from occurring at the electrode surfaces. Instead, it is the movement of ions present in the solution that provides the basis for the measurement. Ions migrate toward the oppositely-charged electrode, changing direction when the polarity of that electrode changes. The magnitude and phase of this alternating ion current are measured, and often just the ac magnitude is used for quantitation.

Fundamental relationships exist between solution conductivity and ion type and concentration; however, these are not used in practical quantitative methods. Calibrations are performed, and it is generally the change in alternating current magnitude that occurs during a defined reaction time, rather than its absolute value, that is used for quantitation.

1.4. RADIOCHEMICAL MEASUREMENTS

Radioactive isotopes of the elements contain unstable nuclear configurations due to their numbers of neutrons, and undergo radioactive decay, to form more stable products. Many radioactive elements have been used as labels in bioassays, including isotopes of iodine, carbon, phosphorus, sulphur and hydrogen. While the use of radioisotopes has been decreasing with the introduction of alternative, less hazardous labels, radioisotopes are still used in a number of methods.

Radioactivity is characterized by the type of decay as well as the half-life of the radioactive element. The major types of radiation are called alpha (α), beta (β) and gamma (γ). Of these, the emitted β and γ forms of radiation are useful, due to the elements used in bioassays. Isotopes of carbon (^{14}C), phosphorus (^{32}P), sulphur (^{35}S) and hydrogen (^{3}H) emit β radiation, which consists of high-energy electrons. These electrons are detected by scintillation counting. Radioactive iodine (^{125}I) is also used as a tracer in bioassays and metabolic studies; it emits γ radiation, which has no mass or charge, but is detectable using a Geiger counter.

The half-life of a radioisotope is the time required for half of the atoms to decay to their more stable products, and this value is a characteristic constant for each radioisotope. For the elements of interest in bioassays, the half-lives are: 5715 years (^{14}C), 14.3 days (^{32}P), 87.2 days (^{35}S), 12.3 years (^{3}H) and 59.9 days (^{125}I).

1.4.1. Scintillation Counting

This method involves the capture of β particles in a liquid or solid scintillator. The function of the scintillator is to convert these high energy electrons into light that is detected using a photomultiplier tube or photodiode. Liquid scintillators are solutions that usually contain more than one organic species that can both absorb the energy of the β particle and then emit light at a characteristic maximum wavelength. Solid scintillators consist of these types of molecules dispersed in an otherwise transparent solid block.

Depending on their energies, β particles can penetrate a few centimetres to several metres into a surrounding medium, and their capture results in the excitation of scintillator molecules, which release this energy as photons, as they return to their ground states.

Modern instruments for scintillation counting employ coincidence detection, in which two light detectors are used. The detectors are placed in different locations near the scintillator, and a signal is registered only when both detectors simultaneously provide a nonzero signal. This arrangement has greatly improved both the sensitivities and the detection limits of scintillation-based bioassays, since random non-analyte signals are minimized.

1.4.2. Geiger Counting

Geiger counters are used to detect γ radiation, which has a lot of energy, but no mass or charge. This device, called a Geiger-Mueller tube, consists of a housing that

contains polarized inner electrodes, an anode and a cathode. The tube is filled with a mixture of ionizable gases such as argon and neon. The cathode is a hollow metal cylinder of large area, while the anode is typically a wire located in the centre of the cylindrical cathode.

The γ radiation penetrates the housing of the tube and collides with the glass or metal walls or electrodes, causing the ejection of electrons that collide with the gas molecules, causing their ionization due to the loss of outlying electrons. These reactions result in the formation of cations and electrons in the gas phase, and because of their positive and negative charges, migrate to and are captured by the electrodes. This results in a measurable current in an external circuit. These devices are very sensitive, and produce current spikes as random collections of individual events are detected. The amplified audio version of these current spikes are the common experience of those familiar with these devices.

Different Geiger counter designs are used for detection of γ radiation in air and for immersion in liquid samples.

1.5. SURFACE PLASMON RESONANCE

This method is used to detect and follow binding reactions that occur on a surface. It is largely used for research on the thermodynamic and kinetic properties of association/dissociation reactions, but practical applications to quantitative binding assays and biosensors have been suggested.

This is an optical detection method, in which the angle of reflection of a polarized and generally monochromatic light beam is influenced by the refractive index of a very thin layer of material bound to a very thin (≈50 nm) layer of a metal, which is often gold. The surface-bound layer is on one side of this thin, planar film, while a glass prism is present on the other side.

The principles of SPR are illustrated in Figure 1.3. Light is directed onto one side of the prism. It refracts at the air-glass interface, partially reflects off of the gold film, and travels out the other side of the prism, refracting once again at the glass-air interface. A position-sensitive light detector, such as a photodiode array or a CCD camera, is used to monitor both the intensity and angle of the exiting light beam.

The surface plasmon phenomenon involves the excitation of electrons present at the interface between the conducting material (the gold film) and an insulating layer bound to the surface opposite the prism. As the incident light angle is varied, a critical angle is reached at which light is absorbed at the interface, creating surface plasmons. At this surface plasmon resonance angle, a minimum is detected in the intensity of reflected light. The angle at which the minimum occurs is dependent upon the refractive index of the layer of surface-bound material.

As the incident light angle is scanned, light intensity is measured, and data are plotted as intensity against incident angle. From these data, the resonance angle is determined. Since the resonance angle depends on the refractive index of the surface-bound layer, and this depends on what is bound to the surface, any change in angle indicates a change in the nature of the layer.

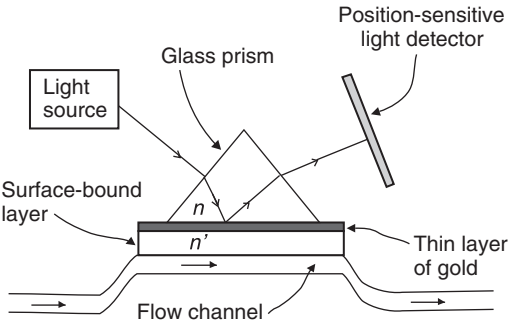

Figure 1.3. Instrumental principles for simple surface plasmon resonance measurements.

Association/dissociation reactions, with one binding partner immobilized on the gold surface (often with the aid of a thin polymer coating to allow covalent binding of one reactant), have been studied using this method. The change in resonance angle can be of the order of 0.1 degree, but instruments are able to measure changes as small as ten microdegrees. Because the resonance angle is sensitive to potentially fluctuating experimental parameters such as temperature and flow rate, a second complete flow cell with a second chip and detection system is used to provide a reference measurement, and the differences between the sample and reference measurements are recorded.

These sensor chips, as they are called, are often employed with a specially designed flow cell, to allow the continuous flow of buffer containing introduced quantities of the solution-phase binding partner across the surface opposite the prism. Binding reversal, and regeneration of the original surface conditions, allows multiple measurements using the same chip.

Challenges with this method include the need for strict temperature control and the elimination of nonspecific binding (adsorption) of solution-phase species that do not interact with the surface-bound binding partner, but stick to the surface of the chip. Another present obstacle involves the expense of the chips, but manufacturing advances may occur to lower these costs, making this transduction method more practical for routine assays.

1.6. CALORIMETRY

Calorimetry involves the measurement of heat. Bio(chemical) reactions may be either exothermic or endothermic, and involve the release or absorption of heat. These reactions result in an increase or decrease in the temperature of the reaction medium. The rate of change of temperature with time is proportional to the rate of the reaction. Two possibilities exist for measurement: temperature change can be monitored as a function of time, using a thermally-jacketed reaction cell, or the reaction medium can be maintained at constant temperature with measurement of the energy required to do this, again as a function of time.

These methods can be used to study protein-ligand interactions, protein unfolding and denaturation, DNA denaturation, DNA/protein/lipid-drug interactions, drug-delivery agents, the incorporation of drugs into nanoparticles, lipid phase transitions, antimicrobial drug mechanisms of action as well as drug purity and thermal stability. Their introduction into the bioanalytical fields has resulted from improvements in sensitivities and detection limits, because limited quantities of biomolecules are commonly available for these studies.

1.6.1. Differential Scanning Calorimetry (DSC)

Older DSC instruments used adiabatic measurements, meaning that heat transfer between samples and their environments was absent or at least minimized by thermal jackets. Since then, it has been shown that non-adiabatic systems can be used to improve baseline stability and reproducibility.

Modern DSC instruments employ microcells such as capillaries, so that no temperature gradients exist across a sample (large surface area/volume ratio), and employ a power compensation method to monitor the difference in power required to maintain sample and reference cells at the same temperature. Temperature is scanned and, since equilibrium conditions are implied by the equations used for subsequent calculations, slower scan rates are preferred (a very slow scan rate for DSC is 0.1 °C/min). There is a tradeoff, however, since slower scan rates yield smaller signals and require more sensitive measurements. Some instruments can simultaneously monitor multiple samples against the same reference solution.

The difference in power required to maintain sample and reference solutions at the same temperature is used to calculate the excess heat capacity of the sample over the reference solutions, as a function of temperature, as temperature is scanned. The excess heat capacity is plotted against temperature in a thermogram (or thermoanalytical curve). The thermogram exhibits a negative-going peak for an exothermic reaction, and a positive-going peak for an endothermic process.

Simple two-state transitions, with no cooperativity, yield flat baselines with symmetric peaks in their DSC thermograms. The peak maximum occurs at T_m, the transition midpoint temperature, and the area under the peak yields the enthalpy of the transition (ΔH). Because, at T_m, the Gibbs free energy (ΔG) of the transition is zero, and since $\Delta G = \Delta H - T_m \Delta S$, entropy ($\Delta S$) can also be calculated as the ratio of $\Delta H/T_m$.

Most biological systems exhibit sloped baselines with asymmetric peaks, due to multiple states during the transition and/or cooperative association/dissociation processes. Many studies have investigated the deconvolution of the resulting thermograms, and some have applied the peak width at half-height, $\Delta T_{1/2}$, to determine the number of states, their fractional occupancies, and the cooperativity of a given overall transition.

Bioanalytical applications of DSC are generally concerned with changes in T_m values with, for example, solvent conditions, pH values and concentrations of analytes, rather than the absolute values of Tm and its associated thermodynamic

parameters. Comparisons of different drugs, for example, can be used to determine which drug binds more strongly to a given protein, causing stabilization (or destabilization) of its fully folded state. Similarly, stabilization of double-stranded DNA towards denaturation by intercalating anticancer drugs can be studied by DSC. DSC applications do not extend to the determination of concentrations of biomolecules.

1.6.2. Isothermal Titration Calorimetry (ITC)

Instruments for ITC are designed to maintain sample and reference solutions at constant temperature during the course of a titration. Sensitive temperature difference measurements and an electronic feedback loop are used to introduce or remove heat from the sample solution during the course of the titration as reagent is added. Instruments measure the input of power as a function of time, and values are corrected for the volume of titrant added to the sample cell. Each aliquot of reagent causes a spike of power, and the integration of a power spike with respect to time results in a value for the heat liberated or evolved as a result of each reagent addition. Cumulative integration of spikes allows a titration curve to be plotted, from which thermodynamic parameters and reaction stoichiometry can be determined.

Applications of ITC include the determination of association constants over a very wide range (10^2 to $10^{12}\,M^{-1}$), reaction mechanisms, and the study of enzyme kinetics and inhibition.

1.7. AUTOMATION: MICROPLATES, MULTIWELL LIQUID DISPENSERS AND MICROPLATE READERS

Automation of bioanalytical methods has developed along with individual reaction and measurement principles. Many methods have been adapted for use with microplates and their associated hardware/software systems to allow simultaneous reactions and measurements on large numbers of samples.

Microplates are disposable, molded, hollow plastic trays of approximate dimensions $12.8 \times 8.5 \times 1.4$ cm, that possess wells in which reactions occur. The number of wells per plate may be as few as 6 or as many as 1536 (6, 24, 96, 384, and 1536 well plates are in use; while 3456 and 9600 well plates are being developed) with corresponding well volumes of several mL down to tens of nL. Wells are arranged in a 3×2 pattern: e.g. a 6-well plate is arranged as 3 columns by 2 rows, a 96-well plate (the most commonly used at this time) has 12 columns by 8 rows, and a 1536-well plate has 48 columns and 32 rows. Ultra-high-density microplates currently under development have 72×48 wells (3456) and 120×80 wells (9600). Wells are usually circular in top view, but may be square for certain applications (for sample storage, square wells provide better seals with their corresponding covers). Wells are about 1 cm deep and may be flat-bottomed, hemispherical or conical. Disposable well inserts are available for lower density microplates, for purposes that include filtration and tissue culture. For microplate filtration, special swinging-bucket rotors are used for centrifugation of balanced pairs of microplates that contain filtration inserts.

Polystyrene is the most common material used for microplates, but many other materials are also used, depending on the surface properties and detection methods desired. Polystyrene may be modified by the addition of either titanium dioxide, for opaque white microplates compatible with luminescence detection, or carbon, for opaque black microplates used for fluorescence detection. Scintillating microplates have also been developed. Inner well surfaces may be modified either for deactivation towards nonspecific adsorption or to provide reactive surfaces for chemical modification.

In addition to polystyrene, polypropylene, polycarbonate and cyclo-olefin materials are also available. Polypropylene microplates are used for applications involving wide temperature variations (e.g. −80 to +100 °C). Polycarbonate is very inexpensive and is useful for less extreme temperature variation (e.g. +4 to +90 °C). Cyclo-olefin microplates have been introduced for measurements involving ultraviolet light, since the other materials are transparent to visible light in their unmodified forms but absorb ultraviolet radiation. Polystyrene, polypropylene and cyclo-olefin microplates are manufactured by injection-molding, while the softer polycarbonate microplates are vacuum-formed.

Microplates can be used in conjunction with various robotic equipment and multichannel detectors for automated, simultaneous multi-sample assays, commonly referred to as high-throughput screening (HTS).

Pneumatic liquid dispensers, calibrated to accurately and precisely deliver equal volumes of solution to each well, are available to simultaneously initiate and stop reactions. Multichannel aspirators and plate washers are also used, along with robotic devices for agitation (reagent mixing) as well as microplate movement, stacking and incubation.

Microplate readers, or detectors, are mostly based on the optical principles of absorption, fluorescence and bio(chemical) luminescence spectroscopy. Measurements may be sequential or parallel, and may involve complex monochromators or simple wavelength selection filters. The introduction of ultra-high-density plates with 3456 and 9600 wells has been limited largely by the pace of development of microplate readers that have adequate quality and speed.

At present, a state-of-the-art, high-end microplate reader from one manufacturer allows multiple modes of measurement (absorbance, fluorescence intensity, fluorescence polarization, time-resolved fluorescence and luminescence), contains a dual monochromator system, and allows kinetic measurements with 3456-well microplates. For comparison, inexpensive microplate readers for UV-visible absorption only are based on removable filters (a specific filter is purchased for the wavelength of interest) and are useful for routine/repetitive measurements at this wavelength, for example, if the same label is used in assays for routine screening of a library of enzyme inhibitors, or if an absorbance-based assay is used for routine medical laboratory testing. Robotic options (liquid dispensing, stacking, incubating) as well as software options are generally available.

Advances in this field are fast-paced, and are due largely to demand from the pharmaceutical industry, for which the ultra-HTS screening of compound libraries is of significant importance. The reader is advised to use the internet to determine the

current state-of-the-art in microplates, associated robotics, microplate readers and data analysis software.

1.8. CALIBRATION OF INSTRUMENTAL MEASUREMENTS

For any instrumental measurement, calibration of the instrument's signal is critical. While inputs, or independent variables (such as wavelength calibration for monochromators or applied potential calibration for amperometric devices) are calibrated less frequently, the calibration of output signals that depend on analyte concentration must be done at least daily, and with each batch of unknown samples. Calibration involves the determination of the mathematical relationship between an instrument's output signal and analyte concentration. This relationship may be linear (allowing standard linear regression) or non-linear (and possibly requiring curve-fitting software, e.g. for enzyme kinetics or antibody-antigen reactions). Accepted standard methods for calibration are detailed below.

1.8.1. External Standards

This method involves the preparation of a series of separate solutions containing the analyte at different, precisely (and accurately) known concentrations. These solutions are examined by the instrumental method to be employed, and the instrumental signal at each concentration is obtained. A plot of signal against [analyte] (or log [analyte]) is obtained. The equation for the dependence of signal on concentration is obtained, and this equation is used to determine the analyte concentrations in the unknown solutions.

Two important points must be made regarding the use of this popular calibration method. The first is that measurements must be made on a "blank" solution, containing no analyte, since the obtained signal will not necessarily be zero. A reagent-blank solution should be used for this purpose, in which all assay reagents are present in the assay buffer at their initial concentrations. There may be a constant or time-dependent signal that results from the reagents themselves or the background decomposition of the reagents that contributes to all signals obtained with standard and unknown samples. Signals for the reagent-blank solution are subtracted from signals obtained with standards and unknowns. Replication, or multiple measurements made on identically-prepared solutions, significantly improves precision, when calibration curves are prepared using the average measurement for the reagent blank and for each standard solution; average values for the unknown solutions are then also used.

Special consideration should also be given to matrix matching. The sample matrix consists of everything present in the sample solutions other than the analyte. The unknown samples may be present in a solution that contains many other chemical or biochemical species, and these may influence the instrumental signal obtained for the unknowns but not the signals for the standard solutions. All available knowledge should be applied to the preparation of standard analyte solutions in a matrix

that resembles the unknown solutions. Validation methods, such as the parallelism test (Chapter 17), can be used to determine whether this is a significant problem, and, if so, either increased dilution of unknown solutions or the use of the standard additions method is recommended.

1.8.2. Internal Standards

This method involves the intentional addition of a new (bio)chemical compound to all standards and unknowns. This compound must not already be present in either standard or unknown solutions, it should be structurally similar to the analyte, and it should behave in similar physical and chemical ways. The quantity of internal standard added to standard analyte solutions and unknown solutions should be the same, and the additions are made before any sample preparation stages.

Calibration data for each solution are collected by measuring the unique signals for each of the two compounds (the analyte and the internal standard). For example, the two compounds may absorb light maximally at different wavelengths, and an absorbance reading would be acquired at each of these wavelengths. For each solution, the signal ratio is then calculated as the signal from the analyte divided by the signal for the internal standard. This value is then plotted against [analyte] to produce the calibration curve. The signal ratios for each of the unknown solutions are then calculated and used to determine the unknown analyte concentrations.

The purpose of the internal standard method is to improve both precision and accuracy. During the sample preparation steps employed prior to the instrumental measurement, the internal standard, if well chosen, behaves identically to the analyte (for example, in precipitation, centrifugation or solvent extraction steps). Thus, if only 80% of the original quantity of analyte remains for instrumental measurement, a similar percentage of internal standard also remains (the exact percentages are not critical, as long as they are constant). During measurements, slow fluctuations in instrumental parameters are common and these may include light source power and light detector sensitivity (for optical methods), flow rate variations (chromatographic methods), voltage or temperature fluctuations (electrophoresis) and ionization source efficiency (mass spectrometry). The use of the signal ratio, rather than the absolute signal from the analyte, these fluctuations are cancelled, or at least minimized, leading to improved precision and lower detection limits.

The so-called “isotope dilution” method, commonly used in mass spectrometry, is an application of internal standards that has been given a special name. A stable or unstable isotope of an element in the compound under investigation is added at known concentration to an aliquot of the analyte solution. Separate mass spectra are obtained for an aliquot of the original solution and for the “spiked” solution. The signal intensity (or intensity change if the spike isotope was already present at low natural abundance) is used to determine the signal change that results from the spike concentration, while the ratio of signals from the main analyte signal to the spike isotope signal is used to determine the analyte concentration. This is an example of a single-point internal standards method, and is not recommended unless the linearity

of the signal magnitude with concentration has already been verified for both the analyte and the spike isotope.

1.8.3. Standard Additions

While this method is more cumbersome than either the external or internal standards methods, it is necessary for accurate quantitation of analytes that interact with matrix components present in the unknown solutions. Instrumental measurements often generate signals that correspond to only the "free" forms of the analyte, rather than the total concentrations, because physical properties often change if the analyte is bound to a matrix component. As examples, cholesterol exists in both free and protein-bound forms, many drugs associate strongly with albumin and other serum proteins, and environmental toxins may bind very strongly to humic and other natural materials present in environmental samples. Generally the total concentration of the analyte is the desired quantity, rather than just the free form. Properties that may change upon binding include extractability during sample preparation, optical properties (absorption and/or emission wavelengths), diffusion coefficients, reactivities in chemical or enzymatic assays, abilities to bind with antibodies in immunoassays as well as chromatographic and mass spectrometric parameters.

The standard additions method was introduced to eliminate these problems from accurate analyte quantitation. In this method, a series of solutions are prepared, each containing the same volume of the unknown solution. Increasing volumes of a standard solution, containing a high concentration of the same analyte, are added to each solution in the series, beginning with an addition of zero standard solution and ending with a high enough quantity to be equal to or greater than the quantity present in the unknown aliquots. All solutions are then diluted to the same final volume. Upon mixing, the added analyte behaves in the same way as the analyte originally present, and will bind to matrix components in the same way, since it is the same (bio)chemical species. Thus the [free]/[bound] ratio will be the same for the added analyte as it was for the analyte originally present.

The instrumental signal, which results from only the free form, is then measured for each of the solutions, and this signal is plotted against the added analyte concentration. Ideally, a linear dependence is observed with a distinctly non-zero y-intercept (because analyte is present in the unknown aliquots that are present in each solution).

The equation for the line is then determined, and the x-intercept, which is a negative concentration value, is determined by setting y to zero and calculating x using the regression equation (the x-intercept is the negative of the y-intercept divided by the slope). This value represents the concentration of analyte (after dilution) that would be subtracted from the unknown solution in order to achieve a signal of zero. The positive of this value, corrected for dilution, represents the concentration of analyte present in the original unknown.

Once linearity has been established for a given standard additions method, a less cumbersome single-point standard addition method can be used. In this method only the non-spiked and a single spiked solution are used. While the principles are the same, linear regression is not used with this method. Effectively, the y-intercept is the signal obtained for the non-spiked sample, while the slope is calculated from the signal change divided by the concentration change due to the single spike. Replication is recommended to ensure accuracy.

1.9. QUANTITATIVE AND SEMI-QUANTITATIVE MEASUREMENTS

1.9.1. Exact Concentration

When the analyte is naturally occurring in the sample of interest, it is often the exact concentration (and time-based fluctuations) that is of interest. In this case, a quantitative value, with uncertainty, is the desired result. Calibration methods, whether by linear regression or by other curve-fitting methods, provide a value with its associated uncertainty for the analytical result.

The uncertainty is an important parameter that varies with the method and with the analyte concentration: for example, if analyte concentrations are near the detection limit of the method, very high relative uncertainties are observed. Statistical methods must be used for all quantitative assays, and these are discussed in detail in Chapter 20.

1.9.2. Positive or Negative Result

Many bioanalytical methods and devices are designed as screening tools that are intended to provide a preliminary result regarding whether an analyte is present or absent. The desired result is not a quantitative number, but a yes/no answer. These semi-quantitative methods are based on measurements for which a cutoff value is predetermined. The signal magnitude is used to determine whether the analyte is or is not present, according to the cutoff value.

During method development, using samples with known analyte concentrations, the cutoff value is adjusted to minimize false negative results (for most applications). True positive and true negative results are obtained when the method correctly predicts the presence or absence of the analyte. False positives occur when the analyte is present below the cutoff value but the method indicates its presence above this value. False negatives occur when the method predicts the absence of the analyte, yet it is present above the cutoff concentration.

These types of tests, in a medical context, are used to screen for pathogens or antibodies to pathogens, in large sample populations. Positive results in a screening test lead to further, more detailed testing, while negative results lead to no further investigation. For this reason, the elimination of false negatives is a priority, and cutoff values are adjusted for this purpose.

SUGGESTED READING

D. C. Harris, *Quantitative Chemical Analysis*, 8th Ed., W. H. Freeman, New York, 2011.
D. A. Skoog, F. J. Holler, and S. R. Crouch, *Principles of Instrumental Analysis*, 6th Ed., Thomson Brooks/Cole, Belmont, CA, 2007.
M. H. Chiu, and E. J. Prenner, *J. Pharm. Bioallied Sci.* **3**, 2011, 39-59.
M. W. Freyer, and E. A. Lewis, *Methods Cell Biol.* **84**, 2008, 79-113.

PROBLEMS

1. In quantitative measurements, replication is needed to ensure that measured values really represent results from samples and standards (i.e. they are not statistical outliers), and to allow averaging of values obtained for each preparation. There is a tradeoff with reagent consumption as well as the time needed for preparation and measurement for each sample.

(a) How many replicate measurements should be made to ensure the reliability of results: 2, 3, 6, 10 or 20?

(b) Considering the number chosen for (a), and the use of a 96-well microplate with five analyte concentrations for external calibration as well as a blank solution, how many unknown samples can be examined per plate?

2. Most quantitative measurement methods employ a "reagent blank" as one of the samples along with calibration standards and unknowns. The reagent blank contains all of the components of the other samples, except the analyte (often distilled water or buffer is used instead of an aliquot of analyte solution). Typically, a small but nonzero average value is obtained for replicate reagent blank measurements. How is this value used during the processing of values obtained for standards and unknowns (for example, when fluorescence measurements are used to quantitate a fluorescent analyte)?

3. Measured absorbance values should fall within the range 0.1–2.0 to ensure good measurement precision with most spectrophotometers. An analyte that absorbs maximally at 360 nm with a molar absorptivity of 6600 M^{-1} cm^{-1} will be examined using a 1 cm path length cuvette. What is the corresponding concentration range for this analyte? Assume that the reagent blank gives a very small signal (almost zero).

4. A researcher wants to follow the progress of a biochemical reaction using an instrumental method. For what kinds of reactions might calorimetry be preferred over optical measurements (e.g. absorbance, fluorescence or luminescence)?

5. External standard calibrations are preferred in quantitative measurements because they allow examination of many samples using one calibration curve. Give two reasons why a standard addition calibration method might be initially preferred over the external standards method, considering possible sample components. If results from the two calibration methods are obtained and compared, under what conditions might external standards be used for subsequent samples?