Life Sciences

Spectrophotometry of Biological Macromolecules

Contents

Introduction

The life sciences are the grouped sciences that involve the scientific study of organisms. While biology remains the main focus of the life sciences, technological advances in molecular biology and biotechnology have led to broad interdisciplinary fields. Applications from fields such as bio-engineering has seen the life sciences grow considerably, as the benefits to clinical and pharmaceutical, and food and environmental industries become increasingly apparent.

Ultraviolet (UV) and Visible (VIS) spectrophotometry has become the method of choice in most laboratories concerned with biological macromolecules. Biological macromolecules such as proteins and nucleic acids absorb light in the UV region of the electromagnetic spectrum. Absorbance measurements using UV are favoured for their speed and the ability to recover the sample, however they are relatively inaccurate and serve as estimates only. In spite of this they are commonly used for measuring concentrations, detecting conformational changes, ligand binding, and following enzyme reactions.

Many laboratory processes require that known quantities of biological molecules be used in downstream process, as well as validation and yield analysis of the products themselves. Which is reflected in the ubiquity of spectrophotometers in life science environments.

Beer-Lambert Law

Beer-Lambert Law defines the relationship between the absorbance by a sample, and the properties of that sample.

$$A = \varepsilon c l$$

Where A is the absorbance, which has no units, although it is often referred to in absorbance units (AU). ε is the molar attenuation coefficient of the sample medium ($M^{-1}cm^{-1}$), c is the molar concentration (M), and l is the pathlength (cm).

The Beer-Lambert equation can be rearranged to supply a generally more useful equation that makes concentration the unknown subject from the known molar attenuation coefficient and pathlength, and the experimentally determined absorbance value.

$$c = \frac{A}{\varepsilon \times l}$$

Nucleic Acids

Nucleic acids are biopolymers composed of nucleotides monomers made of three components; a five carbon sugar, a phosphate group and a nucleobase (Figure 1). They are key components of the 'central dogma of molecular biology' (Crick, 1958). A simplified explanation of the flow of genetic information within a biological system, summarised as DNA makes RNA and RNA makes protein. Where their primary function is the storage and transfer of that genetic information.

Nucleic acids can be characterised into two major groups. Ribonucleic acid (RNA), where the five carbon sugar is a simple ribose, and deoxyribonucleic acid (DNA) where the five carbon sugar is deoxyribose. Additional distinctions are that RNA exists as single-stranded (ss), and typically shorter molecules, in many of its biological roles. Whereas DNA exists as longer double-stranded (ds) or duplex molecules, taking the form of a double helix. Furthermore, RNA incorporates the base uracil (U) while DNA incorporates the base thymine (T). An additional classification of nucleic acids is

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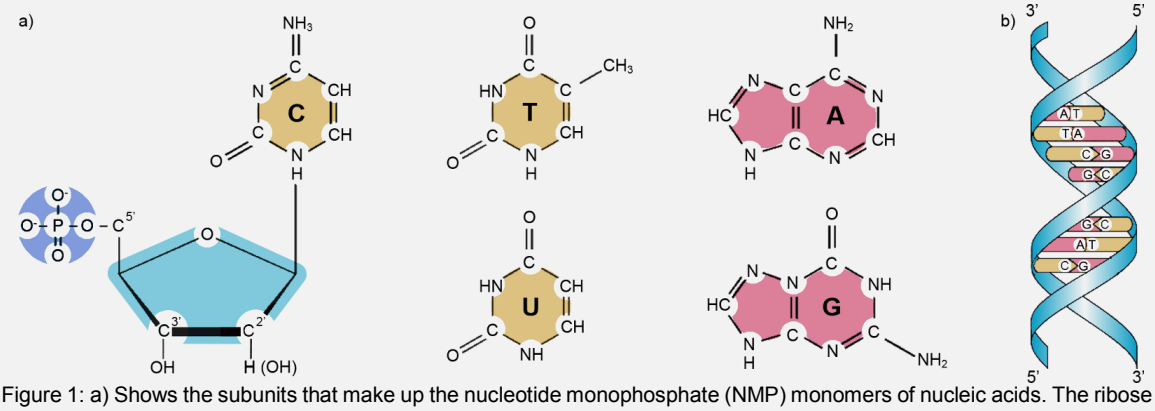

Figure 1: a) Shows the subunits that make up the nucleotide monophosphate (NMP) monomers of nucleic acids. The ribose sugar is shown in blue, and the phosphate group is shown in dark blue. Monomers are joined via a phosphodiester bond that forms between the Carbon$^{5'}$ ($C^{5'}$) phosphate group and the $C^{3'}$ hydroxyl (OH) group to make up the sugar-phosphate backbone. The C2' is linked to a hydrogen (H) in DNA and an OH group in RNA. The nucleobases are shown as yellow for pyrimidines and red for purines. The single letter abbreviations are shown within the 6-cyclic structure and correspond to cytosine (C), thymine (T – DNA only), uracil (U – RNA only), adenine (A), and guanine (G). b) Shows how the bases complement each other in a double-stranded DNA duplex, with each strand's 3' and 5' positions labelled.

oligonucleotides, which are short ssDNA or RNA molecules. Traditionally used to describe nucleic acids less than approximately thirteen nucleotides in length. Longer molecules being termed polynucleotides which fall into the general distinction of DNA or RNA.

Proteins

Proteins are macromolecules made up of one or biopolymers consisting of peptide-linked amino acid residues (Figure 2). They have varied roles within biological systems, such as enzymic, structural, transport and carrier, nutrient and storage, contractile and motile, defense, regulatory, and toxic functions (Jain, Jain and Jain, 2010). Proteins differ from one another principally in their primary polypeptide sequence of amino acids. Which in turn, is dictated by the nucleotide sequence of their genes, and often results polypeptide chains folding into a specific three-dimensional protein, deterining its biological activity.

Nucleic Acid Measurements

The absorbance of UV radiation by nucleic acids is due to the ring structures of the pyrimidine and purine bases. The absorbance maxima (λ_{max}) of the nucleotide monophosphate (NMP) bases and deoxy NMP (dNMP) bases at pH 7.0 range from 252 to 280 nm and 253 to 280 nm respectively (Gerstein, 2002) (Table 1). Rather than having to determine the global absorbance maxima for every polynucleotide of interest, DNA and RNA are considered to absorb strongly at 260 nm.

Base stacking within the hydrophobic core of a double helix, effectively shields the bases from excitation and limits the amount of UV radiation absorbed by the molecule. In dsDNA the bases are stacked, and as result the absorbance is

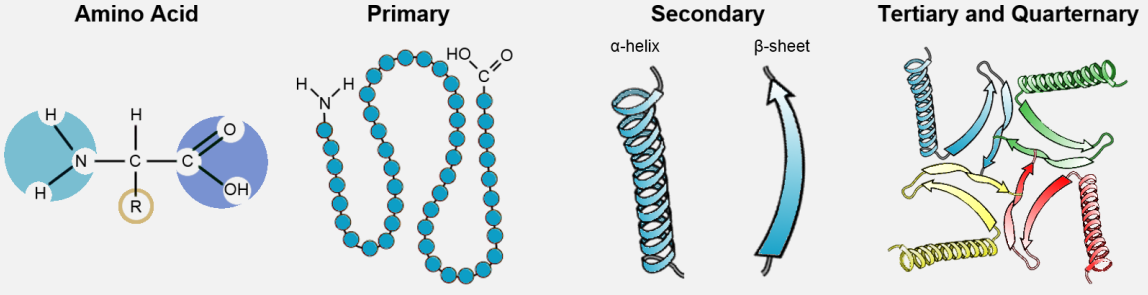

Figure 2: Shows the structure of an amino acid, with the amine group (NH_2) shown in blue, the carboxyl group (COOH) shown in dark blue, and the side chain position (R) shown in yellow. Monomers are joined via a peptide bond that forms between the NH_2 group and COOH group to make up the polypeptide chain. The primary protein structure is a polypeptide chain with the unbound NH_2, termed the N-terminus, and the unbound COOH, termed the N-terminus. The secondary protein structure is defined by the amino acid sequence which can form α-helices and β-sheets, these present hydrophobic and hydrophilic surfaces which cause the protein to fold into its tertiary structure, as shown by the blue subunit, and is stabilised by disulphide bonds between nearby cysteine residues. Quaternary protein structure consists of two or more individual polypeptide chains, or subunits as indicated in different colours, which operate as a single functional unit. The quaternary structure is stabilised by the same non-covalent interactions and disulphide bonds as the tertiary structure.

Table 1: Nucleotide Absorbance Properties

Polynucleotide	Nucleotide	Molecular Weight	λ_{max}
RNA	AMP	347.2 g mol^{-1}	259 nm
	UMP	324.2 g mol^{-1}	262 nm
	GMP	363.2 g mol^{-1}	252 nm
	CMP	323.2 g mol^{-1}	280 nm
DNA	dAMP	331.2 g mol^{-1}	259 nm
	dTMP	322.2 g mol^{-1}	267 nm
	dGMP	347.2 g mol^{-1}	253 nm
	dCMP	307.2 g mol^{-1}	280 nm

proportionally lower to that of ssDNA. Moreover, oligonucleotides which exhibit diminished flexibility about the sugar-phosphate backbone, due to their shorter length, which results in the bases being even more exposed show a proportionally higher absorbance to that of ssDNA.

Quantification

To calculate the concentration of nucleic acids from an absorbance measurement, the molar attenuation coefficient must be known. This can be calculated from the polynucleotide base sequence. Although values vary between literature, representative values (Table 2) can be applied to the nearest neighbour model.

$$\varepsilon = \sum \varepsilon_{Nearest\ Neighbour} - \sum \varepsilon_{Individual\ Bases}$$

For example, to apply the calculation to the DNA sequence 5'-ATGCA-3', the sequence would be separated into the nearest neighbour pairs AT, TG, GC, and CA. It should also be noted that the 5' and 3' bases are excluded from the individual bases calculation.

Within the life science sector it is conventional to quantify biological molecules in weight per volume rather than molar concentrations. Therefore it is mathematically simpler to convert the molar attenuation coefficient to a mass attenuation coefficient (g^{-1}cm^{-1}), also described as the absorbance for a 0.1 % weight per volume sample ($Abs\ 0.1\ \%$), by simply dividing a molecules molar attenuation coefficient by its molecular weight (MW) in g mol^{-1}. Substituting this into the rearranged Beer-Lambert equation would give the nucleic acid concentration in 0.1 % weight per volume units, for example mg/mL.

$$mg/mL = \frac{A_{260}}{Abs\ 0.1\ \% \times l}$$

However it is common to expect the concentration to be in the 0.0001 % weight per volume range, and to work in microliter volumes. Consequently it is typical to express nucleic acid concentrations in ng/µL. To achieve this the absorbance can be multiplied by one thousand.

$$ng/\mu L = \frac{(A_{260} \times 1000)}{Abs\ 0.1\ \% \times l}$$

In the interest of mathematical simplicity, a concentration factor can be determined by dividing one thousand by the mass attenuation coefficient. This process results in a concentrations factor (k) that can be easily applied to the absorbance, to give relevant concentration units.

$$ng/\mu L = \frac{A_{260} \times k}{l}$$

Calculating the molar attenuation coefficient using the nearest neighbour model, although relatively accurate and several online resources being available to perform the calculation on the operators behalf, is protracted and still only provides an estimate. Therefore common concentration factors are often applied to nucleic acids absorbance measurements. They are 50 for DNA, 40 for RNA, and 33 for oligonucleotides. As a result, a DNA sample measuring 1 AU at 260

Table 2: Nearest Neighbour and Individual Base Molar Attenuation Coefficients

		3' Position			
		A	**T**	**G**	**C**
		15400 M^{-1}cm^{-1}	8700 M^{-1}cm^{-1}	11500 M^{-1}cm^{-1}	7400 M^{-1}cm^{-1}
5' Position	**A**	27400 M^{-1}cm^{-1}	22800 M^{-1}cm^{-1}	25000 M^{-1}cm^{-1}	21200 M^{-1}cm^{-1}
	T	23400 M^{-1}cm^{-1}	16800 M^{-1}cm^{-1}	19000 M^{-1}cm^{-1}	16200 M^{-1}cm^{-1}
	G	25200 M^{-1}cm^{-1}	20000 M^{-1}cm^{-1}	21600 M^{-1}cm^{-1}	17600 M^{-1}cm^{-1}
	C	21200 M^{-1}cm^{-1}	15200 M^{-1}cm^{-1}	18000 M^{-1}cm^{-1}	14600 M^{-1}cm^{-1}

nm with a 1 cm pathlength, equates to a concentration of 50 ng/µL.

$$\frac{1 \times 50}{1} = 50\ ng/\mu L$$

Should a downstream application require molar concentrations, the ng/µL concentration can be divided by the polynucleotides molecular weight (MW) in g mol^{-1}, to give the millimolar (mM) concentration.

$$\frac{ng/\mu L}{MW} = mM$$

Purity

The relative absorbance of nucleic acids and proteins shows that nucleic acids absorb many times more than proteins do. For example, just 50 µg of DNA in one millilitre will absorb 1 AU at 260 nm, but 1.5 mg (thirty times more) of bovine serum albumin (BSA) in one millilitre is required to absorb 1 AU at 280 nm. Therefore, a relatively small peak at 280 nm can mean a large concentration of contaminating protein which needs to be identified.

Calculating the ratio between the absorbance at 260 and 280 nm has become common practice for determining any degree of protein contamination.

$$\frac{Nucleic\ Acid\ Purity}{from\ Protein} = \frac{A_{260}}{A_{280}}$$

Although some debate exists (Glasel, 1995), DNA samples with a ratio 1.8 and RNA samples with a ratio of 2.0 are considered free from protein contaminants.

An additional purity ratio between the absorbance at 260 and 230 nm is useful for identifying the presence of contaminants associated with nucleic acid preparations, such as carbohydrates, guanidine hydrochloride (GuHCl) and, or phenol.

$$\frac{Nucleic\ Acid\ Purity}{from\ GuHCl\ and\ Phenol} = \frac{A_{260}}{A_{230}}$$

Pure preparations of nucleic acid have a ratio of 2.0 (Figure 3).

Ratios less that those indicated above suggest that there may be some level of contamination. As a result additional analysis, such as visualisation using electrophoresis, and further purification, such as protein denaturation and phenol-chloroform extraction, should be performed.

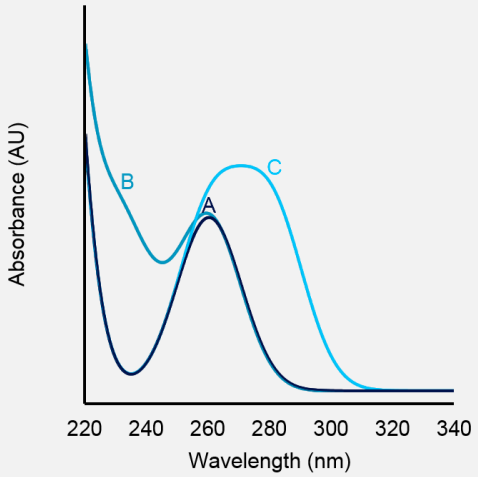

Figure 3: Representative UV Spectra of pure DNA absorbance at 260 nm (A), and with equally absorbing contamination at 230 nm (B) and at 280 nm (C).

Melting Temperature

The increase in absorbance upon disruption of base stacking within DNA or RNA double helices, as described previously, is called hyperchromism. It provides a very sensitive and convenient probe for monitoring strand dissociation and unfolding of double helices.

Duplex nucleic acids are primarily held together by noncovalent hydrogen bonds between the bases, and to a lesser extent hydrophobic and van der Waals interactions between the aromatic rings through base stacking interactions. Such weak interactions are easily broken during heating. This dissociation between the two strands is described as 'melting', and it can be followed by observing

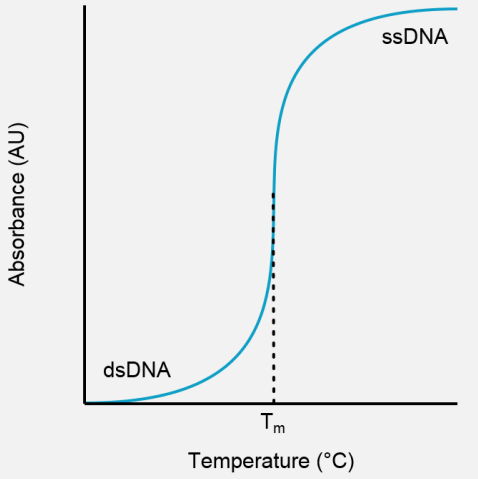

Figure 4: DNA melting curve that shows the change in absorbance with an increasing temperature.

an absorbance increase at approximately 260 nm. A melting curve can be created by plotting the absorbance over temperature. The melting temperature (T_m) is defined as the temperature where half the total change in absorbance is observed (Figure 4).

The melting temperature of duplex polynucleotides depends its G and C base content. GC base pairs form three hydrogen bonds, so are relatively more stable than AT base pairs which only form two. As a result, melting temperature increases with GC content, and furthermore, GC content can be determined from the melting temperature. Long duplex DNA molecules with unequal distribution of its GC content, exhibiting localised resistance to increasing temperature, give complex melting curves and include several melting points. In this cases the melting temperature is defined by the maxima of the first derivative of the melting curve.

Protein measurements

Two aromatic amino acid residues, tryptophan (Trp) and tyrosine (Tyr), absorb relatively strongly at 280 nm with molar attenuation coefficients of 5500 and 1490 $M^{-1}cm^{-1}$ respectively. The disulphide bonds (SS) between cysteine (Cys) residues has a relatively low molar attenuation coefficient of 125 $M^{-1}cm^{-1}$, but still contributes to the global protein absorbance at 280 nm (Figure 5).

Quantification

The global molar attenuation coefficient of a protein can be calculated providing the number (n) of its Trp, Tyr, and SS are known (Pace *et al.*, 1995), using their individual molar attenuation coefficient.

$$\varepsilon = 5500 \times n_{Trp} + 1490 \times n_{Tyr} + 125 \times n_{ss}$$

The individual molar attenuation coefficient above represent average values for each chromophore in a folded protein.

The absorption by amino acids is influenced by their surroundings, and Trp and Tyr undergo a red-shift in nonpolar compared to a polar environments. As a result, native proteins, where

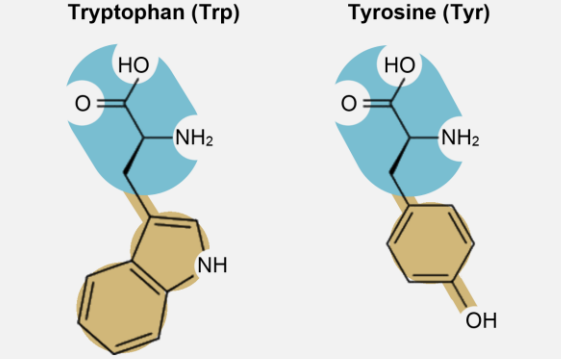

Figure 5: Tryptophan (Trp) and tyrosine (Tyr) residues. The NH_2 and COOH groups that form the polypeptide chain are in blue. The side chains are in yellow.

the residues are exposed to the solvent, and the interior of globular proteins, where residues are buried within the interior, will contribute differently to the degree of absorption. However, such differences are small and typically negligible and the calculation above has an accuracy of about ±5 %. More accurate values can be determined using two identical protein samples with 6 M guanidine hydrochloride (GuHCl). This destabilises the intracellular interactions of the protein, unfolding it into its primary structure. The absorbance of the unfolded protein with solvent-exposed chromophores can then be determined using the previous calculation.

The determined molar attenuation coefficient can then be applied to the rearranged Beer-Lambert Equation. In keeping with the life science industry convention to quantify biological molecules in weight per volume concentrations. The molar attenuation coefficient can be converted to a mass attenuation coefficient ($g^{-1}cm^{-1}$), or $Abs\ 0.1\ \%$, by dividing the molar attenuation coefficient by the proteins molecular weight (MW) in g mol^{-1}, or Daltons (Da) as is commonplace when describing protein molecular weights. Substituting this into the rearranged Beer-Lambert equation gives the protein concentration in 0.1 % weight per volume units (mg/mL)

$$mg/mL = \frac{A_{280}}{Abs\ 0.1\ \% \times l}$$

The $Abs\ 0.1\ \%$ values for bovine serum albumin (BSA), Immunoglobulin G (IgG), and Lysozyme are often used for estimating protein

Table 3: Mass Attenuation Coefficients of BSA, IgG, and Lysozyme

Standard	Calculation	Abs 0.1 %
BSA	$43824\ M^{-1}cm^{-1} \div 66400\ Da$	0.66
IgG	$210000\ M^{-1}cm^{-1} \div 150000\ Da$	1.40
Lysozyme	$37770\ M^{-1}cm^{-1} \div 14307 Da$	2.64

Table 4: Colorimetric Assays

Assay	Active Reagent	Wavelength	Sensitivity
BCA	bicinchoninic acid	562 nm	0.0005 – 2 mg/mL
Biuret (Piotrowski's test)	Biuret reagent	546 nm	5 – 160 mg/mL
Bradford	Coomassie Brilliant Blue dye	595 nm	0.001 – 2 mg/mL
Lowry	Folin–Ciocalteu reagent	750 nm	0.005 – 2 mg/mL

concentrations with low, medium, and high mass attenuation coefficient respectively (Table 3).

Additional common methods of determining protein concentration in 0.1 % weight per volume units exist. A variation on the process outlined above is to use the rearranged Beer-Lambert equation, but multiply the absorbance by the protein molecular weight (MW) in Da.

$$mg/mL = \frac{A_{280} \times MW}{\varepsilon \times l}$$

Another approach is to use the proteins E1 % value, which often appears in literature. This is the mass attenuation coefficient ($g^{-1}cm^{-1}$) of a 1 % weight per volume sample ($Abs\ 1\ \%$), for example 10 mg/mL. This method requires that the absorbance be multiplied by 10.

$$mg/mL = \frac{A_{280} \times 10}{Abs\ 1\ \% \times l}$$

Should a downstream application require molar concentrations, the mg/mL concentration can be divided by the proteins molecular weight (MW) in kDa, to give the millimolar (mM) concentration.

$$\frac{mg/mL}{MW} = mM$$

A number of colorimetric assays are also available for determining protein concentration. The Bradford assay (Bradford, 1976) is particular of the proteins amino acid sequence, so shares some of the limitations of direct UV measurements. However it remains popular due to its speed, high sensitivity, and little interference from reagents. The Biuret and bicinchoninic acid (BCA) (Smith *et al.*, 1985) assays use copper ion interaction with the peptide bonds of the polypeptide chain, as a result they are not subjective of the proteins amino acid sequence. The Lowry assay (Lowry *et al.*, 1951) uses a combination of interactions between copper ions with the peptide bonds and the oxidation of aromatic protein residues. These assays have advantages over direct UV measurements in that they are more sensitive, capable of detecting ng/mL concentrations, and better accuracy. Disadvantages include the sample being non-recoverable, and BSA standard curves needing to be constructed (Table 4).

Purity

The concentration of protein samples contaminated with nucleic acids can be estimated using the Warburg-Christian method (Warburg and Christian, 1942). Where the protein percentage content can be determined from the A_{260}:A_{280} ratio (Table 5).

$$\%P = 100 - \frac{(11.16 \times R - 6.32)}{(2.16 - R)}$$

Where $\%P$ is the percentage protein content, and R is the A_{260}:A_{280} ratio. The concentration of protein also be calculated from the $\%P$ and pathlength (l), although its accuracy is limited to between 0.05 and 0.5 mg/mL.

$$mg/mL = A_{280} \times \frac{1}{l} \times \frac{\%P}{100}$$

Table 5: Warburg-Christian Method

A_{260}:A_{280}	Protein Content (%)	Nucleic Acid Content (%)
0.57	100	0
1.06	95	5
1.32	90	10
1.48	85	15
1.59	80	20
1.67	75	25
1.73	70	30
1.78	65	35
1.91	60	40
1.84	55	45
1.87	50	50
1.89	45	55
1.91	40	60
1.93	35	65
1.95	30	70
1.95	25	75
1.97	20	80
1.98	15	85
1.98	10	90
1.99	5	95
2.00	0	100

Source: Measuring Protein Concentration in the Presence of Nucleic Acids by A280/A260: The Method of Warburg and Christian (2006).

This assumes that 1 AU equals 1 mg/mL, by dividing the absorbance at 280 nm by the proteins mass attenuation coefficient ($g^{-1}cm^{-1}$) it can made be more specific to the protein of interest.

$$mg/mL = \frac{A_{280}}{Abs\ 0.1\ \%} \times \frac{1}{l} \times \frac{\%P}{100}$$

A separate simpler method often credited to Warburg and Christian, albeit seemingly incorrect to, can also be used to quantify protein concentration in the presence of nucleic acid contaminants (Layne, 1957).

$$mg/mL = 1.55 \times A_{280} - 0.76 \times A_{260}$$

A similar method that uses the absorbance at 230 nm rather than at 280 nm can also be used (Kalb and Bernlohr, 1977).

$$mg/mL = 0.183 \times A_{230} - 0.076 \times A_{260}$$

Measuring at 230 nm is not Trp or Tyr dependant, instead absorbance at 220-230 nm is due to the polypeptide chain. However, contaminants associated with the absorbance at 230 nm cannot be differentiated.

Protein Unfolding

A protein's secondary structure of α-helices and β-sheets present hydrophobic and hydrophilic surfaces. These form and stabilised the hydrophobic interactions that fold a protein into its native structure, so that hydrophilic sides face out into the aqueous environment surrounding the protein and the hydrophobic sides face into the hydrophobic core of the protein. These hydrophobic interactions, and Van der Waals forces, are relatively weak. However, any Cys disulphide bonds contribute a much stronger structural integrity.

Denaturation, or unfolding, of native proteins can be induced by applying heat (melting) or by adding denaturants such as urea or guanidine hydrochloride (GuHCl), and observed by following the hyperchromicity between 287 and 292 nm. Whether heat or denaturants are used, plotting the absorbance over temperature, or denaturant concentration, produces a melting curve. As with duplex nucleic acid melting curves, the melting temperature (T_m) is defined as the temperature where half the total change in absorbance is observed. That is, where 50 % of the protein is folded and 50 % is unfolded.

Dye Measurements

In addition to the common colorimetric assays described previously for protein quantification. Alternative methods to quantify nucleic acid and protein concentrations is to tag the unknown sample with a dye, and measure the intensity of that dye against a standard curve constructed from a known tagged-molecule. This approach is useful where the concentration is too low to accurately determine using direct UV spectrophotometry, or where there are contaminants absorbing in the UV region.

There are many commercially available dyes, with the most popular belonging to the Cyanine family. Cyanine, or Cy, dyes are synthetic dyes belonging to polymethine group, and the standard range consists of Cy2, 3, 3.5, 5, 5.5, 7, and 7.5. Nucleic acid and protein labelling is usually performed with either Cy3 or Cy5 (Table 6), which can be synthesised to carry one of a number of reactive groups which binds its complimentary target on the molecule of interest.

Table 6: Cy Dyes

Dye	Cy3	Cy5
λ_{max}	550 nm	647 nm
Molar Attenuation Coefficient	150000	250000
Molecular Weight	767	792

Enzyme Kinetics

Many enzyme kinetics studies focus on initial rates of enzyme reactions in what is termed pre-steady-state kinetics. These simple enzyme assays measure the initial, and maximal, rate of enzyme reactions by observing the change in the absorbance between products and reactants. Sometimes referred to as time-course assays, the

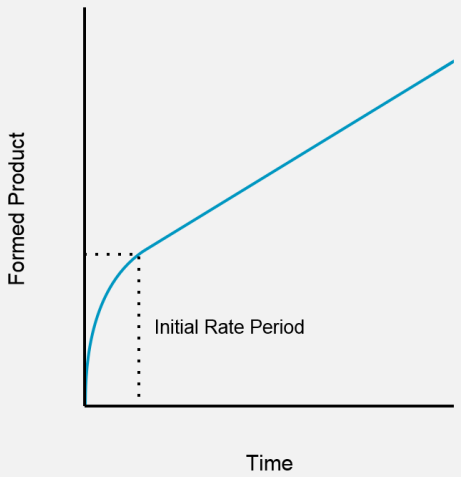

Figure 6: Progress curve for an enzyme reaction. The initial rate is the slope gradient within the initial rate period.

absorbance is plotted over time and the graph produces an initial rate that is closely linear for a short period after the start of the assay (Figure 6). As the assay proceeds and the substrate is consumed, the rate continuously slows. To measure the initial rate, enzyme assays are performed while the reaction is only a few percent towards total completion.

The initial rate period depends on the assay conditions, but can be just a few milliseconds. Where measuring this period becomes impractical, it is possible to measure the complete reaction curve and fit the data to a non-linear rate equation using progress curve analysis (Duggleby, 1995). However, the best-known model for steady-state enzyme kinetics is the Michaelis-Menten model (Michaelis and Menten, 1913). This relates the rate (v), or the change in concentration of formed product ($\Delta[P]$) over the change in time (Δt), to the concertation of substrate ($[S]$).

$$v = \frac{\Delta[P]}{\Delta t} = \frac{V_{max}[S]}{K_M + [S]}$$

Where V_{max} is the maximum rate achieved by the system, at saturating substrate concentration. K_M is the Michaelis constant defined as the substrate concentration for half the V_{max}, and can be thought of as a measure of an enzymes binding affinity to a substrate (Figure 7). The Michaelis-Menten model can only be applied to reactions involving a single substrate, multiple substrate reaction mechanisms such as ternary complex and ping–pong as less commonly applied.

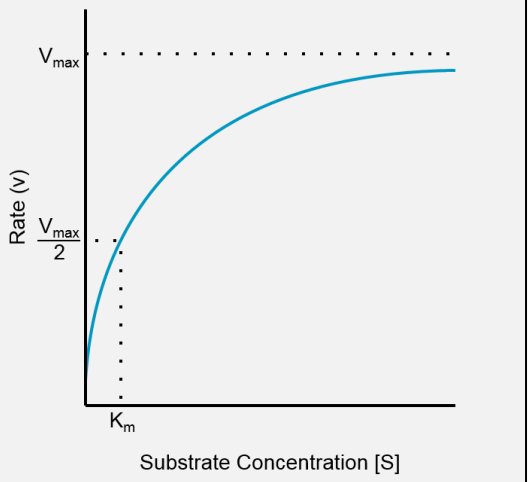

Figure 7: Michaelis-Menten model for an enzyme reaction. The rate, or change in formed product over the change in time, of an enzyme reaction at various substrate concentrations are plotted over those concentrations to find the Michaelis constant (K_m). A protein with a lower K_m has a higher affinity for its substrate than a protein with a higher K_m.

Good Operating Practice

Good laboratory practice should always be applied in the preparation of samples for spectrophotometric assays. The cleanliness of all materials and equipment, especially the sample cuvettes, is imperative, as are weighing and volumetric accuracy.

Further considerations should also be made to assure the accuracy and repeatability of measurements.

Dependence on Environment

Absorbance measurements are influenced by environmental factors such as temperature and buffer composition. As already discussed, temperature can affect the integrity of duplex nucleic acids and the tertiary structure of proteins. Furthermore, refractive index and sample concentration decreases due to the thermal expansion of the solution, and the ionisation of dissociable groups can change.

Changes sample buffer composition can also result in undesirable changes to absorbance measurements. Nucleotide bases, and Trp and Tyr residues can be protonated and therefore are sensitive to changes in pH. Salt in buffer reduces overall relative negativity and therefore decreases the solubility of the molecule of interest. The molecules stability can also be effected by salt concentration. In duplex DNA the negatively charged phosphate groups of the backbone repel one another. This electrostatic repulsion is interfered by counter-ions from dissolved salts. Therefore, duplex stability increases with buffer salt concentration. Some detergents absorb in the UV range, notably IGEPAL, Triton x-100, and NP-9. As a result alternative detergents such as Brij-35, CHAPS, or Tween 20 are recommended. There are also reduced (hydrogenated) Triton x-100 commercial alternatives that reportedly have lower absorbance in the UV range.

Although changes in temperature and buffer composition are best avoided, the combined effects have only a minor influence on absorbance measurements in the absence of structural transitions. However, changes in polarity can shift the absorbance maxima of the molecule of interest, resulting in a high level of variability. A red-shift (increase in absorbance maxima) happens when the excited state is more polar than the ground state, so a polar solvent favours the excited state more than the ground state. A blue-

shift (decrease in absorbance maxima) happens when the excited state is less polar than the ground state so a polar solvent favours the ground state more than the excited state. For example, the absorbance maxima of Tyr is shifted by about 3 nm, from 277nm to 274 nm, when the solvent is changed from carbon tetrachloride to water. Therefore, changes in solvent should always be associated with representative reference samples of buffer minus the molecule of interest.

Solvent selection, as well as potential contamination from upstream processes, should be carefully considered. Water and the less polar alcohols can confidently be used for sample measurements above 190 nm. Whereas chloroform, which is commonly used in DNA extraction (Birnboim and Doly, 1979), can influence the measurement at 260 nm. The wavelength at which the absorbance of the solvent has an unacceptable influence on a measurement is described as the solvent cut-off. Generally two solvent cut-offs are proposed, termed L_0 and L_1. L_0 is the cut-off used in quantitative data collection, and is the wavelength where the solvent has an absorbance of no more than 0.05 AU in a 1 cm pathlength. L_1 is the cut-off used in qualitative data collection, and is the wavelength where the solvent has an absorbance of no more than 1 AU in a 1 cm pathlength (Table 7).

Table 7: Cut-offs for Common Solvents

Solvent	L_0	L_1
Acetone	340 nm	330
Chloroform	260 nm	240
Dimethylsulphoxide	330 nm	285
Ethanol	240 nm	205
Methanol	240 nm	205
2-Propanol	240 nm	205
Water	190 nm	185

Equipment Considerations

Cuvettes used for reference and sample measurements should be optically matched over the measurement wavelength range. Alternatively, a plot of absorbance against concentration can be made, and the intercept at the y-axis can be applied as a correction factor (k) to the Beer-Lambert equation.

$$A = \varepsilon lc + k$$

Successive sample measurements made using the same cuvette should be effectively cleaned, including sample solvent washes and drying. The orientation of the cuvette in the cell holder should remain consistent for continuity of the cuvette surfaces and pathlength. Several processes can contribute to the total attenuation of light as it passes through a cuvette and sample, including reflection, scattering, absorbance, and fluorescence. However, the effects of reflection and fluorescence are restricted to less than significant levels by the use of quality sample cuvettes, matched where possible, careful sample handling, and instrument design.

The ability of an instrument to accurately quantify a molecule of interest in a sample depend upon, among other factors, the natural bandwidth (NBW) of that molecule of interest and the spectral bandwidth (SBW) of the instrument. Generally, a ratio between the SBW and NBW of 0.1 will generate absorbance measurements at 99.5 % accuracy.

$$\frac{SBW}{NBW} \leq 0.1$$

Therefore when measuring large biological macromolecules, DNA has a natural bandwidth of approximate 43 nm, reliable quantitative measurements can be achieved using instrument with a spectral bandwidth of 5 nm.

$$43 \times 0.1 = 4.3\ nm$$

However, qualitative measurements that resolve the 20 nm region between the DNA peak at 260 nm and a potential protein at 280 nm, would require an instrument with a 2 nm spectral bandwidth.

$$(280 - 260) \times 0.1 = 2\ nm$$

Xenon flash lamp are increasingly common in spectrophotometers designed for use in the life science sector. Although improvements in stabilising the flash discharge are notable, some variation between flashes can persist. The effect is most noticeable for low absorbance measurements, but it can be minimised by taking a duplicate reference measurement of the blank sample to prime the lamp, waiting a predefined period of time and taking a second reference measurement. The blank sample is then replaced with the first test sample during the same predefined period of time and the sample is measured. The waiting time can be as short as a few seconds but must allow time to swap samples.

Short pathlength cuvettes, and standalone micro-volume sample platforms are common place in life science laboratories. Short pathlengths allow for measurements at higher concentrations than the standard 1 cm pathlength. However, they are less sensitive and less reproducible at lower concentrations. Therefore the limitations of short pathlength should be considered. Beer-Lambert law dictates that the most linear region of

absorbance correlating to concentration is between 0.1 and 1 AU. Which correlates to reliable DNA concentration range between 5 and 50 ng/µL in a 1 cm pathlength, between 50 and 500 ng/µL for a 0.1 cm (1 mm) pathlength, and between 500 and 5000 ng/µL for a 0.01 cm (0.1 mm) pathlength. In practice, the application of these pathlengths are governed by the molecule of interest. For example, oligonucleotides can achieve concentrations of well above 5000 ng/µL, while samples of larger DNA molecules become viscous at higher concentrations making handling impractical and increasing the risk of aggregation.

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