

CIRCULAR DICHROISM

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Circular Dichroism (CD) is an essential analytical technique used to analyze chirality in molecules through their optical activity. CD spectroscopy measures the difference in the absorption of left and right circularly polarized light in optically active substances. CD signals are observed for optically active (chiral) materials; however chirality can also be induced via covalent bonding to a chiral chromophore or when the chromophore is placed in an asymmetric environment. CD can be applied to a wide variety of molecular structures, but has found favor in the scientific community for the elucidation of macromolecular structure, especially proteins and nucleic acids.

Electromagnetic waves contain electric and magnetic field components that oscillate perpendicularly in the direction of a light beam's propagation. The directionality of these components defines the waves' polarization. In unpolarized light or white light, the electric and magnetic fields oscillate in many different directions. In linearly polarized light, the electromagnetic wave oscillates along a single plane (Figure 1, left), while in circularly polarized light (CPL) two electromagnetic wave planes are at a 90° phase difference to one another and this plane rotates as the light beam propagates (Figure 1).

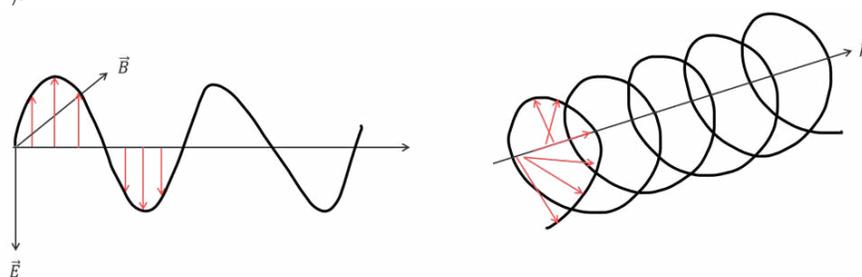


Fig. 1 Linearly (left) and circularly (right) polarized light.

Optically active molecules can be described by their chirality, or the asymmetry in a molecule's structure. Chiral molecules exhibit circular birefringence or optical rotation (OR), where the velocity of the light passing through an optically active medium will differ depending on the medium's refractive index, and rotate the plane of linearly polarized light. The wavelength dependence of OR is called optical rotational dispersion (ORD).

Circular Dichroism spectroscopy exploits the fundamental property described by the Cotton Effect, or the dependence of CD and optical rotation on wavelength (Figure 2). While CD may only be observed in the vicinity of a chromophore's absorption band, ORD may be detected in the absence of an electronic transition since it is only dependent on the unequal refraction of light.

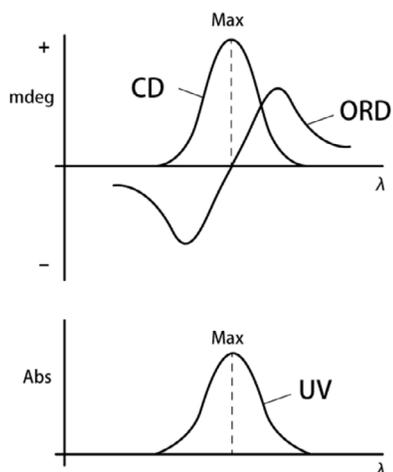


Fig. 2 The relationship between the CD/ORD spectra and the UV spectrum in the absorption wavelength region.

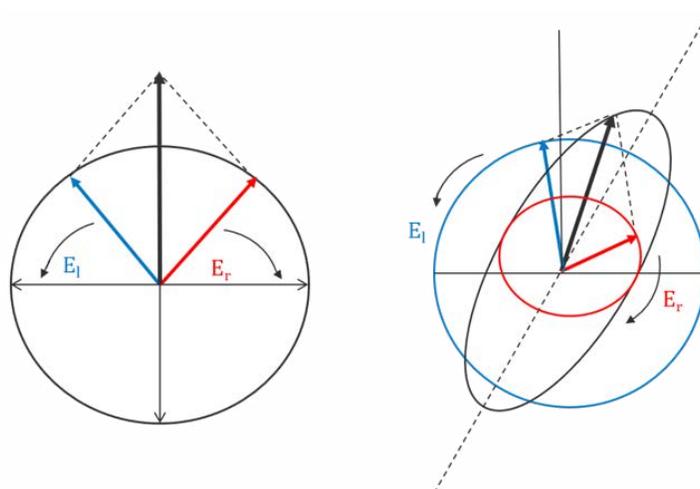


Fig. 3 E_l and E_r components when they are absorbed equally (left), resulting in linearly polarized light, and unequally (right), resulting in elliptically polarized light.

In CD, left-handed CPL propagates through a chiral sample at a different speed than its right-handed counterpart. When a chiral molecule absorbs left- and right-handed CPL to different degrees the resulting electric field vector traces out an ellipse. Since linearly polarized light can be thought of as a superposition of equal intensities of left- (E_l) and right-handed (E_r) circularly polarized light, as the circularly polarized light travels through the birefringent material, the phase relationship between the CPL waves changes and the linearly polarized wave is rotated and the elliptically polarized light is now tilted (Figure 3). This ellipticity (θ) as a function of wavelength defines a circular dichroism spectrum.

This ebook is intended to be a brief overview of some of the key considerations when selecting a CD for your laboratory.

Instrumentation

Photoelastic Modulator (PEM)

In a circular dichroism spectrophotometer, linearly polarized light is created to later produce circularly polarized light. Unpolarized light first passes through a polarizer in which the crystal axes and the orientation of the molecules are aligned. A photoelastic modulator (PEM) is then used to convert the linearly polarized light into circularly polarized light. As seen in Figure 4, the PEM is a piezoelectric element, cemented to a block of fused quartz. When a 50 kHz frequency is applied to the piezoelectric element, it stresses the quartz piece, inducing birefringence and two polarization components. Circular polarization occurs when the phase of an orthogonal electric field deviates by $1/4$ wavelength.

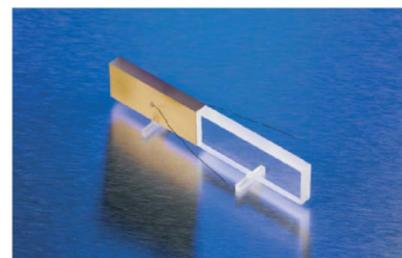


Fig. 4 Photoelastic modulator used to convert linearly polarized light to circularly polarized light.

Nitrogen Purge

The xenon arc lamp creates a substantial amount of UV radiation. When this radiation interacts with oxygen molecules, it creates ozone. Ozone oxidizes the surface of the mirrors in the instrument, decreasing their reflectivity and efficiency to focus light through the monochromator, reducing the S/N. Additionally, oxygen itself is absorbed in the UV region. By purging the monochromator with nitrogen, not only do the mirrors retain their reflectivity and longevity, but the oxygen absorption is decreased, allowing sample data to be acquired further into the far-UV. Figure 5 provides nitrogen purging suggestions.

Designated Wavelength (nm)	Pre-Purge Time (min)	N2 Flowrate for Measurement (L/min)
185	10	2
180	30	5
175	60	15

Fig. 5 Suggested purge times and flow rates and their corresponding wavelength limits.

High Tension Voltage

The photomultiplier tube (PMT) detector has great sensitivity combined with a very wide linear dynamic range to measure both low and high absorbing samples from the far UV to visible regions of the spectrum. The high tension (HT) voltage applied to the PMT is used to amplify the detector's sensitivity. Since different wavelengths have different light energies, the HTV accommodates for fluctuations in the light levels by changing the gain. The gain either increases or decreases the amplitude of the signal depending on the DC voltage output. As shown in Figure 6, when a sample has an optimal concentration, a sufficient amount of light is transmitted through the sample and reaches the detector, keeping the gain low. However, if a sample absorbs too much light, not enough photons will reach the detector and the gain must increase to amplify the signal and therefore the HT voltage will increase. If the HT voltage is equal to or above 700 V, there are not enough photons being sampled by the PMT to measure a reliable or valid CD signal and the data points should not be used. The more photons reaching the detector, the better the S/N so at higher HT voltages, while the HT may be below 700 V, the CD signal may begin to look noisier. Additionally, farther into the far-UV (below 200 nm), less light is available so the HT voltage will also increase at shorter wavelengths.

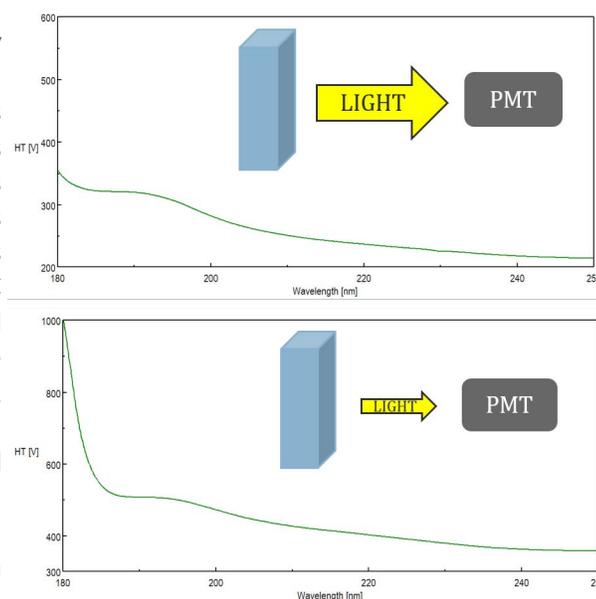


Fig. 6 High tension voltage when a sufficient amount of light is transmitted through the sample (left) and when the light is absorbed too strongly at lower wavelengths (right).

CD is an ensemble technique that provides information on the molecular and electronic structure of chiral chromophores, both quantitatively or empirically. Chromophores that are not intrinsically chiral can be placed in an asymmetric environment or coupled to chiral chromophores, where CD can be induced into these achiral electronic transitions. Most CD experiments require little sample preparation, are easy to perform, and are non-destructive so sample can be recovered. Sample measurements can be acquired in liquid phase or as solid, films, or gels, at low concentrations compared to other molecule structure techniques.

Protein Structure

Secondary Structure

Proteins adopt unique conformations, optimized to perform specific functions. Therefore, the information on the protein structure is paramount to elucidating how proteins function. The far-UV (180-250 nm) region probes the peptide backbone chain, whose phi and psi angles rotate depending on the protein conformation. There are two absorption bands which make up the far-UV CD spectrum: a strong $\pi \rightarrow \pi^*$ around 190 nm and a weaker but broader $n \rightarrow \pi^*$ transition between 210-220 nm, seen in Figure 7.

These two absorption bands give rise to different characteristic bands (Figure 8) that can be deconvoluted to estimate the secondary structure components of the protein under different solution and environmental conditions. While each of the four secondary structure components (α -helical, β -sheet, turn, and unordered) have distinguishing CD spectra, deconvolution is more complex than assigning a band to each component. Secondary structure analyses require curve fitting procedures based on a set of reference spectra with known secondary structure components that is used to estimate the components in the unknown (or sample) spectrum through regression analysis. The aromatic side chain residues and disulfide bonds are also known to contribute to the far-UV spectrum, further complicating estimations. Secondary structure analysis is best used to clarify shifts in CD data. More similar secondary structures, such as β -sheet and turn, can be teased out and structural changes from a majority of sheet to a majority of turn can be confidently confirmed.

Tertiary Structure

The near-UV CD spectrum (250-320 nm) reflects the protein tertiary structure, made up of the aromatic amino acid side chains. The three residues, phenylalanine, tyrosine and tryptophan, exhibit fine structure peaks between 255-270 nm, 275-285 nm, and 290-305 nm, respectively. The number and proximity of the aromatic residues will affect the strength of the CD signal, as well as the disulfide bonds, degree of hydrogen bonding, and rigidity of the chromophores in folded conformations.

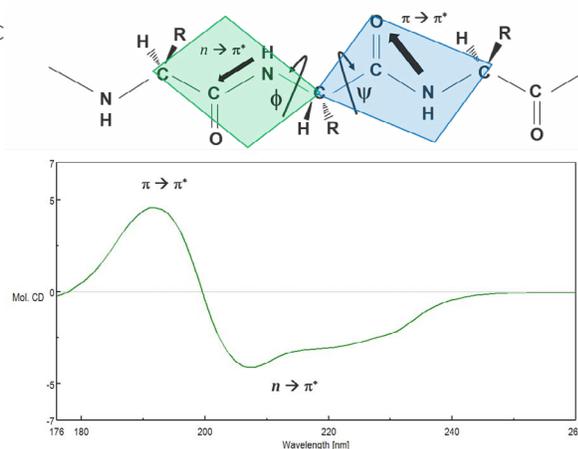


Fig. 7 The peptide bond (top) and the corresponding UV transitions observed in the far-UV CD spectrum (bottom).

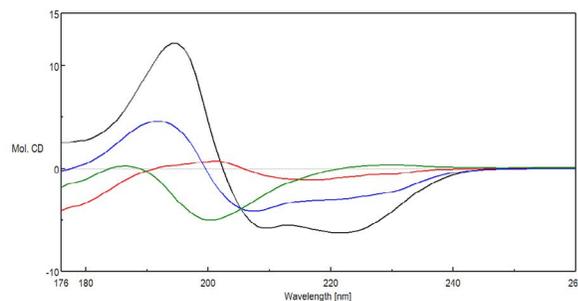


Fig. 8 Far-UV CD spectra of samples with a majority of α -helical (black), β -sheet (red), turn (blue), and unordered (green) components.

DNA Structure

The CD spectra of DNA provides information on the molecule's secondary structure that is dependent on the stacking orientation of the base pairs. While the base pairs themselves are not intrinsically chiral and alone would not exhibit a CD signal, they are covalently bound to the DNA backbone chain composed of sugar moieties. The chirality of the sugar groups induces a CD signal in the base pair's $\pi \rightarrow \pi^*$ transition, observed from 200-300 nm.

Each DNA conformation displays a distinct CD spectrum. The most well known B-form, discovered by Watson and Crick, is a right-handed double helix whose base pairs are perpendicular to the helix. A positive maximum at 290 nm, negative maximum around 245 nm, and a broad positive peak between 260-280 nm are typically seen for B-form structures. The A-form is more compact, right-handed double helix structure that is typically observed for RNA and exhibits a positive peak at 260 nm and a negative peak at 210 nm. The Z-form structure is a left-handed double helix that displays a negative band at 290 nm, a positive peak at 260 nm, and negative maximum \sim 200 nm. G-quadruplex (G4) secondary structures are unique in that they fold into four-strands that are rich in guanine. These quadruplexes can form either parallel or anti-parallel structures depending on the stacking interactions of the guanosine residues. Parallel G4 structures have a positive band at 260 nm while the anti-parallel structures exhibit a negative band at 260 nm and a positive peak at 290 nm.

Thermal Stability

Changes in temperature can affect biomolecular structure and induce aggregation, leading to various disease-related conformations, as well as influence the stability during storage and efficacy of biopharmaceuticals. These studies are used to monitor protein folding/unfolding, as well as the effect of solvents, pH, and ligands on the thermal stability of biomolecules. Thermal stability is assessed either by monitoring single wavelengths at the peak of interest as a function of temperature (Figure 10, top) or by acquiring spectral scans at increasing temperatures (Figure 10, bottom).

Protein folding reversibility is commonly investigated to verify whether a protein can undergo structural changes when heated and revert back to its native form when cooled back down to the initial temperature. If the protein folding is reversible during temperature measurements, then thermodynamic parameters such as the Gibb's free energy, (ΔG) enthalpy (ΔH) and entropy (ΔS) changes, and melting temperature (T_m) can be acquired by fitting the thermal melting curve.

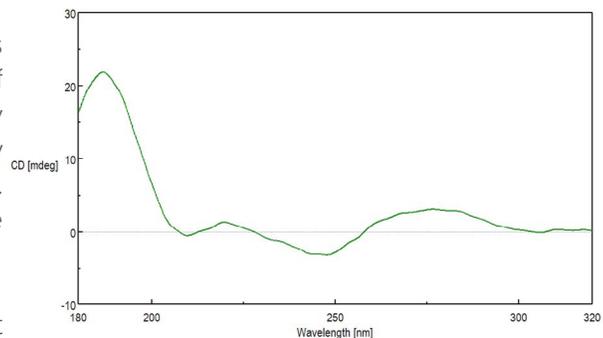


Fig. 9 CD spectrum of DNA derived from bovine thymus.

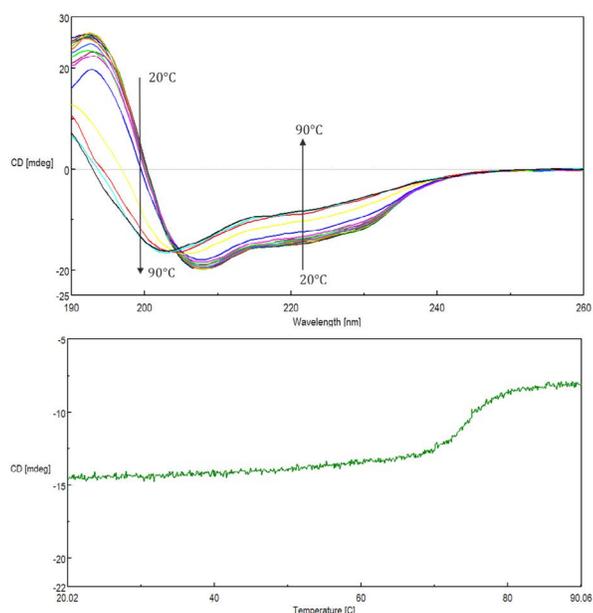


Fig. 10 Thermal melting curve (top) obtained at 222 nm and CD spectral scans (bottom) from 20°C to 90°C measured to evaluate the thermal stability of lysozyme's secondary structure.

Beer's Law: Sample Concentration and Cell Pathlength

CD is an absorption technique based on Beer's Law

$$A = \epsilon lc$$

where A is the absorbance, ϵ is the molar absorptivity constant, l is the cell pathlength, and c is the chromophore concentration. Therefore, the amount of light absorbed by the sample is dependent on concentration and pathlength and critical to obtaining accurate CD data. Usually, the optimal S/N is obtained when the optical density of the absorption maximum wavelength is ~ 1 . Figure 11 additional concentration and cell pathlength guidelines for a variety of chromophores.

Chromophore	Transition	Wavelength (nm)	Concentration (w/v%)	Cell Pathlength (mm)	
-C \equiv C-	$\pi \rightarrow \pi^*$	220-190	0.1	1-0.5	
-C=C-	$\pi \rightarrow \pi^*$	200-185	0.1	0.5-0.1	
-C=C-C=C-	$\pi \rightarrow \pi^*$	300-250	0.01-0.05	10	
C=O, -CHO	$n \rightarrow \pi^*$	350-240	0.1	10	
-C=C-C=O	$n \rightarrow \pi^*$	400-260	0.1	10	
	$\pi \rightarrow \pi^*$	280-200		1-0.2	
-COOH, Lactone, Ester	$n \rightarrow \pi^*$	250-200	0.1	10-1	
S-S	$n \rightarrow \sigma^*$	300-200	0.1	10	
Protein, Polypeptide	Aromatic Amide	$\pi \rightarrow \pi^*$	350-250	0.1	5
			260-185	0.02	1-0.5
			260-200	0.1	
DNA, RNA	$\pi \rightarrow \pi^*$	300-200	0.1	1-10	
Co-chelate Complex	$d \rightarrow d^*$	700-300	0.1	10	
	CT	300-180	0.01	1	

Fig. 11 Common chromophores and their corresponding concentration and cell pathlength requirements.

Conveniently, the sample concentration and cell pathlength can be adjusted to account for absorbance limitations. For example, if the sample absorbance is too large but the concentration cannot be reduced for experimental purposes, the cell pathlength can be shortened to acquire the necessary measurements. Likewise, if only one cell pathlength is available, the sample concentration can be increased to obtain data with a sufficient S/N.

Aside from the pathlength selection, the cuvette or cell material and volume are also important parameters to consider when performing circular dichroism experiments. For measurements below 200 nm, quartz cuvettes are necessary due to their transparency in the far-UV. While most quartz rectangular cuvettes have little to no birefringence and flat baselines, baseline measurements should be performed to ensure no optical artifacts are observed due to the strain on the cuvettes. Additionally, these cell baseline measurements can elucidate the cleanliness of the cell. While demountable cells hold much smaller volumes than the tradition 1 and 10 mm pathlength cuvettes, these cells cannot be used for temperature dependent measurements since they are not completely sealed, resulting in heat lose. It is also important to verify the pathlength of demountable cells after sample as been loaded to ensure the correct pathlength, especially is the molar ellipticity or mean residue molar ellipticity is later calculated. Rectangular microcells are available for small volume measurements and come in a variety of volumes and pathlengths. JASCO also offers two microsampling accessories; a disc for spectral scans using only 2 or 10 μ L and a capillary cell for thermal stability studies.

Solvent Effects

The buffer or solvent the sample is in will also affect the CD spectrum. Solvents used for absorption measurements will typically suffice, however the following conditions should be taken in consideration when choosing a solvent for CD measurements: (1) solubility, (2) transparency in the wavelength range being probed, (3) optically inactive, and (4) sample stability. Condition 2 is especially important for secondary structures studies in the far-UV, since many solvents absorb strongly below 200 nm, as well as denaturing agents such as guanidinium chloride and urea. The addition of salts to buffers will may also increase absorbance and potentially induce scattering, reducing the S/N. Sugar-based formulation buffers not only absorb highly in the far-UV but also have a CD signal and should be avoided for CD measurements.

Solvent	Useable Short Wavelength Limit (nm)		
	1 cm	1 mm	0.1 mm
Ethanol/Methanol (4:1)	~220	~200	
Distilled Water	~185	~180	~175
10 mM Sodium Phosphate		~182	
0.1 M Sodium Phosphate		~190	
0.1 M Sodium Chloride		~195	
0.1 M Tris-HCl		~200	
0.1 M Ammonium Citrate		~220	

Fig. 12 Various buffers and their corresponding wavelength limits in 10, 1, and 0.1 mm pathlength cells.

Digital Integration Time and Scanning Speed

The digital integration time or D.I.T. is the amount of time that the data is integrated over or the length of time the detector collects photons before transferring the signal to the A/D converter for processing. The square root of the D.I.T. is proportional to the signal to noise, so the longer the D.I.T. the better the S/N. Increasing the D.I.T. will have a more substantial effect when a sample's CD signal is small since there is less signal.

The scanning speed determines how quickly the monochromator scans through the specified wavelength range to acquire data points at the specified data pitch. When used in continuous scan mode, the scanning speed must be selected with an appropriate D.I.T. to prevent distortion in the measured spectrum. The following guideline can be used when selecting the D.I.T. and scanning speed

$$D.I.T. \times \text{Scanning speed} < \frac{FWHM}{3}$$

where FWHM is the full width at half the peak height of the target peak. Figure 13 illustrates the effects of the scanning speed and digital integration time on the far-UV CD spectra.

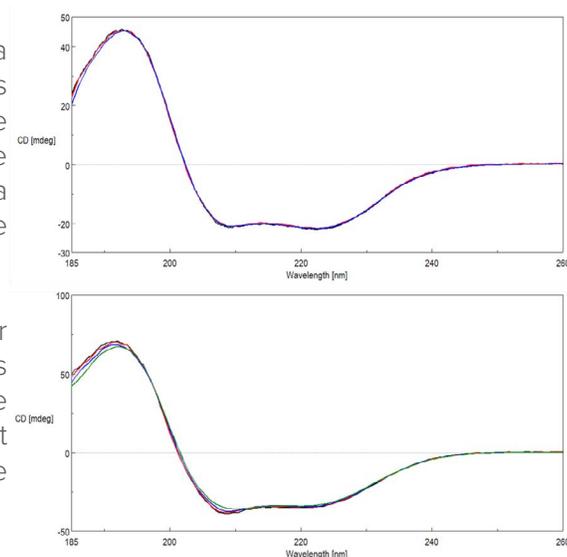


Fig. 13 The effects of D.I.T. (top) and scanning speed (bottom) on the far-UV spectrum. Myoglobin spectra were measured using a 50 nm/min scanning speed and 1 (black), 2 (red), and 4 (blue) second D.I.T. Bovine serum albumin was acquired using a 4 second D.I.T. and 10 (black), 20 (red), 50 (blue), and 100 (green) nm/min scanning speed.

Accumulations

The number of spectral scans that are automatically obtained and averaged together are specified by the accumulations. The spectrum S/N is proportional to the square root of the product of the D.I.T. and number of accumulations. While increasing the number of accumulations will increase the S/N, it will also increase the measurement acquisition time. When selecting the number of accumulations and D.I.T., baseline drift and peak widths should be taken into account.

CD Units

CD spectra are reported as ellipticity, θ and measured in units of mdeg but can easily be converted based on the user's application. Molar absorptivity ($\Delta\varepsilon$) normalizes the circular dichroism spectra based on concentration and pathlength,

$$\Delta\varepsilon = \varepsilon_l - \varepsilon_r = \frac{\Delta A}{c \cdot l} = \frac{\theta}{c \cdot l \cdot 3298}$$

where $\varepsilon_l - \varepsilon_r$ is the difference in the extent the left- (ε_l) and right- (ε_r) circularly polarized light components are absorbed at a given wavelength, ΔA is the difference in absorbance, c is the concentration (mol/L), and l is the pathlength (cm). When analyzing the secondary structure components, the number of amino acid residues in the protein must be accounted for and ellipticity is converted to mean residue molar ellipticity (MRME)

$$[\theta]_{MR} = \frac{\theta \cdot c \cdot l}{R}$$

where θ is the ellipticity (mdeg), c is the protein concentration in mol/L, l is the cell pathlength (cm), and R is the number of amino acid residues in the protein.

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