2.28 Circular Dichroism Spectroscopy

The circular dichroism spectroscopy is a method used to analyze and determine the structure of optically active substances, discriminate active substances from enantiomers, diastereomers, etc by using the phenomenon (circular dichroism) in which the degrees of absorption of left and right circularly polarized lights differ in the absorption wavelength region of active substances.

In this method circular dichroism is measured as the difference of absorbance of left and right circularly polarized lights as follows.

$$\Delta A = A_L - A_R$$

ΔA: Difference of absorbance of left and right circularly polarized lights
A_L: Absorbance of left circularly polarized light
A_R: Absorbance of right circularly polarized light

Also, the difference of molar absorption coefficients for left and right circularly polarized lights can be expressed as the molar circular dichroism as follows.

$$\Delta\varepsilon = \varepsilon_L - \varepsilon_R = \frac{\Delta A}{c \times l}$$

$\Delta\varepsilon$: Molar circular dichroism ($(mol/L)^{-1} \cdot cm^{-1}$)
ε_L: Molar absorption coefficient for left circularly polarized light ($(mol/L)^{-1} \cdot cm^{-1}$)
ε_R: Molar absorption coefficient for right circularly polarized light ($(mol/L)^{-1} \cdot cm^{-1}$)
c: Concentration of an optically active substance in solution (mol/L)
l: Path length (cm)

The following unit can also be used as the unit indicating circular dichroism.

Dissymmetry factor (g factor):

$$g = \frac{\Delta\varepsilon}{\varepsilon}$$

ε: Molar absorption coefficient

Molar ellipticity:

In some apparatuses, circular dichroism is expressed in units of ellipticity (º). In such a case, the molar ellipticity $[\theta]$ is calculated using the following equation.

$$[\theta] = \frac{\theta}{10 \times c \times l}$$

$[\theta]$: Molar ellipticity ($^{o} \cdot cm^2/dmol$)
θ: Value (mº) of ellipticity calculated by apparatus
c: Concentration of an optically active substance in solution (mol/L)
l: Path length (cm)

Molar ellipticity is related with molar circular dichroism by the following equation.

$$[\theta] = 2.303\Delta\varepsilon \frac{4500}{\pi} \approx 3300\Delta\varepsilon$$

Molar circular dichroism and molar ellipticity are often used for analysis of peptides, proteins and nucleic acids. In this case, mean residue weight, which is the molecular mass divided by the number of monomeric residues, is used in the calculation of molar concentration(c).

$$\text{Mean residue weight} = \frac{\text{molecular mass}}{\text{number of amino acid residues or nucleotide residues}}$$

Mean residue weight is 100 – 120 (generally 115) for peptides and proteins, and is about 330 as sodium salt for nucleic acids.

1. Apparatus

A circular dichroism spectrophotometer is used. A xenon lamp is used as the light source. Light from the light source is polarized at the time when being split by a double monochromator equipped with a crystal prism, resulting in monochromatic linearly polarized light. The slit at the exit of the monochromator eliminates extraordinary light. The monochromatic linearly polarized light is passed through a photoelastic modulator to be alternately modulated into left and right circularly polarized lights at a constant frequency and is irradiated to a sample.

After the light that has passed through a sample to be tested reaches a photomultiplier tube, the light is divided into two electrical signals and amplified. One is the direct current signal, V_{DC}, which reflects the light absorption of the sample. The other is the alternating current signal, V_{AC}, which occurs when the sample has circular dichroism and has the same frequency as the modulation frequency of the photoelastic modulator. The phase of the direct current signal indicates the sign of the circular dichroism (+ or -), and the magnitude of the amplitude indicates the intensity of the circular dichroism. Here, V_{AC}/V_{DC} is proportional to the difference of the absorbances for left and right circular polarized lights, ΔA. Generally, the wavelength range measured by a circular dichroism spectrophotometer is about 170 to 800 nm, but some apparatuses can measure at a wider wavelength range.

2. Methods

Set temperature, wavelength, path length and sample concentration for measurement. Dissolve a sample in an appropriate solvent, place it in a cell, and measure. In the sample preparation, confirm the influence of impurities on the spectrum, the structural change of the sample depending

on the concentration, the absorption of the solvent itself, and the influence of the solvent on the sample structure. Attention should be taken for the optical path length of a sample cell, especially when the optical path length is short. Furthermore, it should be noted for the absorption of light by a sample because it may reduce a signal reaching a detector.

2.1. Identification test

Specify molar circular dichroism or molar ellipticity along with the wavelength at which it is maximum. The identity of a substance can be confirmed when the molar circular dichroism or molar ellipticity at the specified maximum wavelength of the substance to be confirmed meets this specification. Or, when the spectrum of a sample is compared with the reference spectrum of the substance to be confirmed or the spectrum of the reference standard, and both spectra give the same intensity of molar circular dichroism or molar ellipticity at the same wavelength, their identity can be confirmed mutually.

2.2. Analysis of secondary structure

For peptides and proteins, specific spectra appear in the far ultra-violet region. The secondary structure of peptides and proteins can be estimated by measuring the spectrum below about 250 nm. Furthermore, it is possible to estimate the three-dimensional structure from the near ultra-violet spectrum. However, it should be noted that circular dichroism measurement observes the average property of a whole molecule. For a α-helix structure, negative maxima appear generally at 208 nm and 222 nm and a positive maximum between 191 nm and 193 nm, for a β-sheet structure, a negative maximum appears between 216 nm and 218 nm and a positive maximum between 195 nm and 200 nm, and for an irregular structure, a negative maximum appears between 195 nm and 200 nm. Methods for analyzing the proportion of secondary structures from a circular dichroism spectrum include a method using a calculation formula and a method using a database. It can also be calculated by multivariate analysis. Whenever any method is used, the method used for the calculation is specified in the test method.

3. Verification of the performance of apparatus

A wavelength-calibrated apparatus is used, and the performance of the apparatus is verified using a sample with quality suitable for the measurement of circular dichroism and with known $\Delta\varepsilon$.

3.1. Accuracy of circular dichroism

Calibrate the accuracy of circular dichroism with a substance with known $\Delta\varepsilon$, such as isoandrosterone, ammonium *d*-camphorsulfonate, etc. (substances recommended by the apparatus manufacturer may be used). When using isoandrosterone, weigh exactly 10.0 mg of isoandrosterone, and dissolve in ethanol (99.5) to make exactly 10 mL. When the circular dichroism spectrum of the prepared solution is measured in the range of 280 nm to 360 nm using a cell with a path length of 10 mm, $\Delta\varepsilon$ at 304 nm is +3.3.

3.2. Linearity of modulation

Calibrate the linearity of modulation with a substance with known $\Delta\varepsilon$, such as ammonium *d*-camphorsulfonate (substances recommended by the apparatus manufacturer may be used). When using ammonium *d*-camphorsulfonate, weigh exactly 6.0 mg of ammonium *d*-camphorsulfonate and dissolve in water to make exactly 10 mL. When the circular dichroism spectrum of the prepared solution is measured in the range of 185 nm to 340 nm using a cell with a path length of 1 mm, $\Delta\varepsilon$ at 290.5 nm is +2.2 to +2.5 and $\Delta\varepsilon$ at 192.5 nm is –4.3 to –5.