2.05 Size-Exclusion Chromatography

(2.05 サイズ排除クロマトグラフィー)

Size-exclusion chromatography is a separation technique of liquid chromatography, which separate molecules in solution based on their size. It is used for the determination of the molecular mass of high-molecular mass compounds such as polysaccharides, nucleic acids, proteins and chemically synthesized polymers, determination of the molecular mass distribution, purity tests and so on. A method using an aqueous solvent as the mobile phase for water-soluble high-molecular compounds is also called gel filtration chromatography. A method using an organic solvent as the mobile phase is also called gel permeation chromatography. Here methods using an aqueous solvent as the mobile phase are described. The principle of the separation is the same when using an organic solvent as the mobile phase.

1. Principle of separation

Components to be tested are separated according to the easiness of entry into the pores of the packing material of the column. Molecules larger than the largest pore move rapidly through the spaces between the particles of the packing material without entering the pores, and elute at the position of the retention volume of an unretained compound (V_0) of the column. Molecules smaller than the pores enter into the pores depending on their sizes, and smaller molecules elute later because they enter deeper into the pores. Small molecules that can freely enter all pores elute last at the position of a completely permeated compound, or the total mobile phase volume (V_t). The elution position of a molecule will be influenced not only by molecular mass but also by the structure of the molecule, solvent and interaction between the molecule and the packing material, etc.

2. Apparatus

A liquid chromatograph is usually used. A porous packing material is used for a column. As packing materials, silica particles whose surface are coated with hydrophilic modification, or cross-linked hydrophilic polymers are used. Since measurable molecular mass range differs depending on the pore size of a packing material and its distribution, select an appropriate column. In order to expand the measurable molecular mass range, a column may be connected with another column having the different target molecular mass range. As a mobile phase, a buffer solution etc. is used. It is important to select appropriately a mobile phase to suppress interaction other than the principle of size exclusion between a packing material and a component to be tested. Adjustment of pH, addition of salt, etc. may be useful to suppress electrostatic interaction between a packing material and a component to be tested, and addition of an organic solvent (methanol, acetonitrile, etc.) may be useful to suppress hydrophobic interaction. The flow rate of a mobile phase, column temperature, sample injection volume, and the concentration of a sample solution should be set appropriately because they affect the separation. As a detector, an ultraviolet-visible spectrophotometer, a differential refractometer, a static light scattering detector, a dynamic light scattering detector, an evaporative light scattering detector, etc. are used. Usually, the molecular mass of a component to be tested is determined by comparing the elution position with molecular mass standards, but when using a static light scattering detector, the molecular mass of molecules in an eluate can be obtained directly.

3. Procedure

Follow Liquid Chromatography *<2.01>*.

4. Measurement of molecular mass

When determining molecular mass, average molecular mass or molecular mass distribution by size exclusion chromatography, measure a sample solution and molecular mass standard solutions prepared using appropriate molecular mass standards under the same test conditions, and determine as follows, unless otherwise specified. The molecular mass standards should have the same physical properties as a component to be tested. The obtained molecular mass value depends on the molecular mass standards used and analysis conditions, and is a relative value to the molecular mass standards.

4.1. Molecular mass of a monodisperse component

A molecular mass calibration curve is prepared by plotting the relationship of retention volume (or retention time) and the logarithmic value of molecular mass labelled on molecular mass standards. The molecular mass will be determined from the molecular mass calibration curve for the retention volume (or retention time) obtained from the chromatogram of a sample solution. Usually, the molecular mass of a component to be tested should be within the range of the molecular mass calibration curve.

4.2. Average molecular mass of a polydisperse component

A molecular mass calibration curve is prepared from chromatograms obtained from molecular mass standard solutions. A chromatogram obtained from a sample solution is divided, and the molecular mass of each eluted fraction is obtained from the molecular mass calibration curve. The concentration or amount of the component in each fraction is determined, and the number-average molecular mass (M_n), mass-average molecular mass (M_w) and dispersity (d) of the sample are calculated by the following equations.

The dispersity may be an indicator of the width of molecular mass distribution.

$$M_n = \frac{\Sigma M_i N_i}{\Sigma N_i} = \frac{\Sigma C_i}{\Sigma \frac{C_i}{M_i}} = \frac{1}{\Sigma \frac{w_i}{M_i}}$$

$$M_w = \frac{\Sigma M_i^2 N_i}{\Sigma M_i N_i} = \frac{\Sigma C_i M_i}{\Sigma C_i} = \Sigma w_i M_i,$$

$$d = \frac{M_w}{M_n}$$

M_i: Molecular mass of i-th fraction
C_i: Concentration of i-th fraction
N_i: Number of molecules in i-th fraction
w_i: Mass fraction of i-th fraction ($w_i = \frac{M_i N_i}{\Sigma M_i N_i} = \frac{C_i}{\Sigma C_i}$)

4.3. Molecular mass distribution

As a distribution curve showing molecular mass distribution, an integral molecular mass distribution curve plotting the logarithmic value of molecular mass on the abscissa and the integral value of mass fraction on the ordinate, and a differential molecular mass distribution curve plotting the logarithmic value of molecular mass on the abscissa and the slope of the integral molecular mass distribution curve determined at each molecular mass on the ordinate, are used.

Specifications for molecular mass distribution can be shown in a form depending on the purpose, such as mass average molecular mass, dispersity, mass fractions of molecules in a specific molecular mass range.

5. Points to consider on system suitability and changes in operating conditions

Liquid Chromatography <*2.01*> is applied to points to consider on specifications of system suitability and changes in operating conditions.