Appendix IV (B): Chromatography – High-Performance Liquid Chromatography (HPLC)

HPLC is a separation technique consisting of a solid stationary phase and a liquid mobile phase. The sample is injected through an injector and carried into the column by the mobile phase, the components are separated on the stationary phase and pass through the detector in succession, a chromatogram is recorded.

(1) **Preparation of test sample** – Powder the CMM sample and pass through a No.2 sieve before analysis. The quantity of the sample to be powdered should be of at least five times as much as those needed for the analysis.

(2) **General requirements for the apparatus** – Set up the stationary and mobile phases of the HPLC as specified in the individual monograph. One of the most commonly used packing material is ODS chemically bonded to silica. Ion exchange resins are used for ion exchange chromatography and porous silica or polymers are used for size exclusion chromatography. The column is usually maintained at room temperature and an UV photometer is used as a detector.

The types of stationary phase, mobile phase and detector as specified in the individual monograph should not be varied. Other parameters may be varied to fit for the performance of the system suitability test when necessary.

(3) **System suitability test** – This is to test the suitability of the instruments according to the requirements prescribed in the individual monograph. By using specified chemical reference substances, adjust the following parameters to comply with the requirements specified in the individual monographs, i.e. to match the *n* value, the repeatability, the *R* value and the *T* value of the column.

(a) **Number of theoretical plates of the column (*n*)** – The *n* value is a measure of the column efficiency. It should not be less than the value specified in the individual monograph. The *n* value is calculated by using the following equation –

$$n = 5.54 \left(\frac{t_R}{W_{h/2}} \right)^2$$

Where t_R = the retention time of the marker peak in the standard solution or analyte peak in the test solution,

$W_{h/2}$ = the peak width at half-height of the marker peak in the standard solution or analyte peak in the test solution.

(b) **Repeatability** – The repeatability is expressed as an estimated RSD of at least five replicate injections of the standard solution. The RSD of the peak area and the retention time should comply with the requirements specified in the individual monograph.

(c) **Resolution factor (*R*)** – To ensure the accuracy of quantitative analysis, the *R* value (Fig. 1) of the analyte peak with the adjacent peak must be larger than 1.5, unless otherwise specified. The *R* value is calculated by using the following equation –

$$R = \frac{2(t_{R2} - t_{R1})}{W_1 + W_2}$$

Where t_{R1} and t_{R2} = the retention times of two adjacent peaks 1 and 2, respectively,
W_1 and W_2 = the widths of two adjacent peaks 1 and 2, respectively.

(d) **Tailing factor (*T*)** – It is necessary to inspect the *T* value (Fig. 2) of the peak, especially when using the peak height method. It should comply with the requirement specified in the individual monograph. The *T* value is calculated by using the following equation –

$$T = \frac{W_{0.05h}}{2d_1}$$

Where $W_{0.05h}$ = the peak width at 0.05 of the peak height,
d_1 = the distance between the perpendicular line passing through the peak maximum and the leading edge of the peak at 0.05 of the peak height.

(4) **Quantitative procedure** – Set up the HPLC system according to the procedures described in the manufacturer's manuals. Under the recommended HPLC conditions, establish the calibration curves by injecting an appropriate amount of standard solutions of a series of concentrations into the HPLC system for analysis. Identify the analyte peaks in the chromatogram of the test solution by comparing their retention times with those of the peaks of the chemical reference substances in the chromatogram of the standard solution obtained under the same HPLC conditions as specified in the procedure. Alternatively, spike an appropriate amount of chemical reference substance in one of the analyzing samples to verify the identified peak.

Prepare a 5-point calibration curve by plotting the peak areas of the chemical reference substance against the corresponding concentrations (in milligram per litre) of the standard solutions. Obtain the slope, y-intercept, the regression equation and the r^2 value from the calibration curve. With the calibration curve

of the corresponding chemical reference substance, calculate the concentration (in milligram per litre) of the analyte in the test solution by using the following equation –

Diode array detector (DAD)

$$\text{Concentration of the analyte} = \frac{A - I}{m}$$

Where A = the peak area of the analyte in the test solution,
I = the y-intercept of the 5-point calibration curve,
m = the slope of the 5-point calibration curve.

Evaporative light scattering detector (ELSD)

$$\text{Concentration of the analyte} = e^{[\text{Ln}(A)-I]/m}$$

Where A = the peak area of the analyte in the test solution,
I = the y-intercept of the 5-point calibration curve,
m = the slope of the 5-point calibration curve.

Calculate the percentage content of the analyte in the sample by using the following equation –

$$\textit{Content (\%) of the analyte} = \frac{C \times V \times D}{10000\ W}$$

Where C = the concentration, in mg/L, of the analyte in the test solution,
D = dilution factor, if any,
V = the final make-up volume, in mL, of the test solution,
W = the weight, in g, of the sample used for the preparation of the test solution.

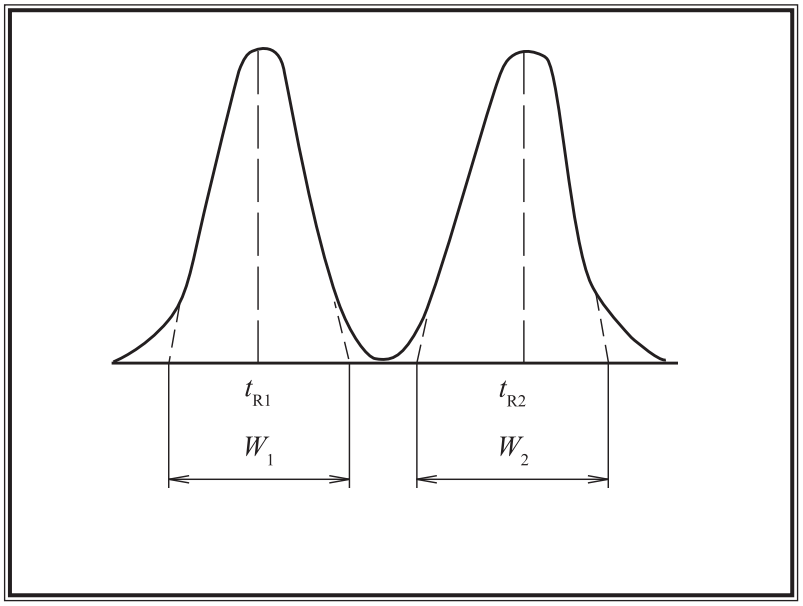

Figure 1 Parameters for calculation of resolution factor (*R*)

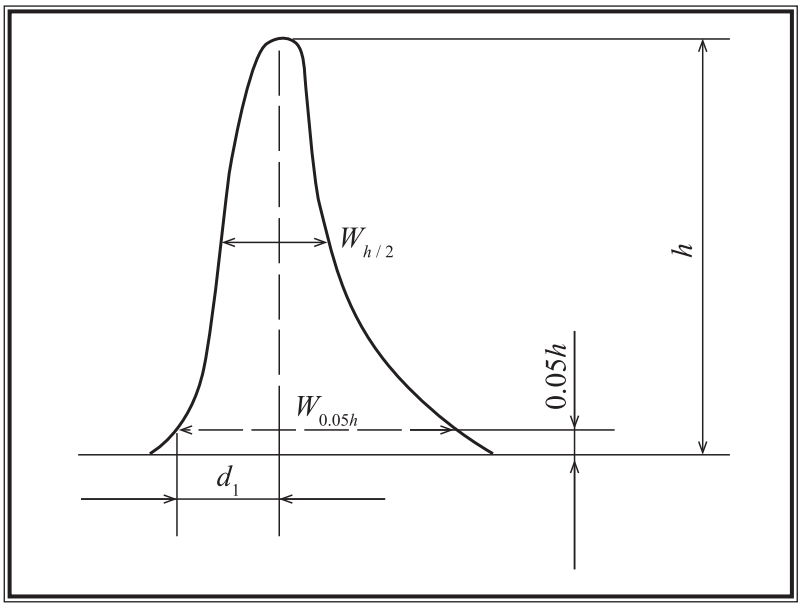

Figure 2 Parameters for calculation of tailing factor (*T*)