## Appendix IV(A): Chromatography - Thin-Layer Chromatography (TLC)

TLC is a separation technique in which a stationary phase consisting of an appropriate material is spread in a uniform thin layer on a support (plate) of glass, plastic or aluminum film. Standard and test solutions are deposited separately on the plate and the components are separated by the developing solvent systems. For the identification of CMM, separated spots obtained from the test solution are compared with the corresponding spots obtained from the chemical reference substance(s) in the chromatogram.

(1) **Apparatus and materials –**

(a) **TLC Plates** – The most commonly used coated plates are silica gel G, silica gel $GF_{254}$, silica gel H and silica gel $HF_{254}$. Diatomaceous earth, diatomaceous earth G, aluminum oxide, aluminum oxide G, microcrystalline cellulose and microcrystalline cellulose $F_{254}$ etc., can be used as well. Coated plates with the size of 10 x 5 cm; 10 x 10 cm; 10 x 15 cm; 20 x 10 cm or 20 x 20 cm are commonly used.

(b) **Application devices** – Micropipettes, micro-syringes, calibrated capillaries or other suitable application devices can be used for the proper application of solutions to the plates.

(c) **Developing chamber** – A tank of size suitable for the plates, with a tightly fitting lid and with a flat bottom or twin trough is usually used.

(d) **Spray reagents** – Spray reagent for the detection of spots is specified in the individual monograph.

(e) **Ultraviolet (UV) light source** – An emitting light source in the UV range is used for the examination of spots in the chromatogram.

(2) **Procedure** –

(a) **Saturation of the developing chamber** – Unless otherwise specified, carry out the chromatography in a saturated chamber. To achieve saturation, pour into the developing chamber of a sufficient amount of the developing solvents, replace the lid and allow it to stand for at least 1 h at room temperature. If necessary, line the inner walls of the developing chamber with two filter paper strips of the same dimensions as the wall, the lower edges of the filter papers should be immersed in the developing solvents.

(b) **Applying the standard and test solutions** – Apply separately the prescribed volumes of the standard and test solutions in small portions to obtain spots (less than 3 mm in diameter) or bands on a line parallel to, and 15 mm from, the lower edge of the plate. Pay attention not to apply spots or bands nearer than 15 mm from the sides of the plate and no disturbance of each other should occur.

(c) **Developing a chromatogram** – Place the plate in the chromatographic tank after the solvent has evaporated from the applied solutions, ensuring that the sample line are 5 mm above the surface of the developing solvents. Then cover the chamber tightly with a lid. Remove the plate from the chamber when the developing solvents have moved over the distance as prescribed in the individual monograph. Dry the plate and visualize the chromatogram in visible light and/or UV light as specified in the individual monograph.

(d) **Interpretation of the chromatogram** – Compare the principal spots or bands observed from the test solution with the corresponding spots or bands observed from the standard solutions. For positive identification, the sample must give spots or bands with chromatographic characteristics, including the colour and the $R_f$ value, similar to those of the chemical reference substances when observed in visible light and/or UV light as specified in the individual monograph.

The $R_f$ value is defined as the ratio of the distance from the point of application to the centre of the spot to the distance travelled by the solvent front from the point of application:

$$R_f = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent front}}$$

## 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 be not 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 repeat 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. 37) 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. 38) 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 –

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

Where $W_{0.05}$ = 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 different 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 –

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

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.

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

$$\textit{Content (\%) of the analyte} = \frac{C \times V \times D}{10{,}000\ W}$$

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.

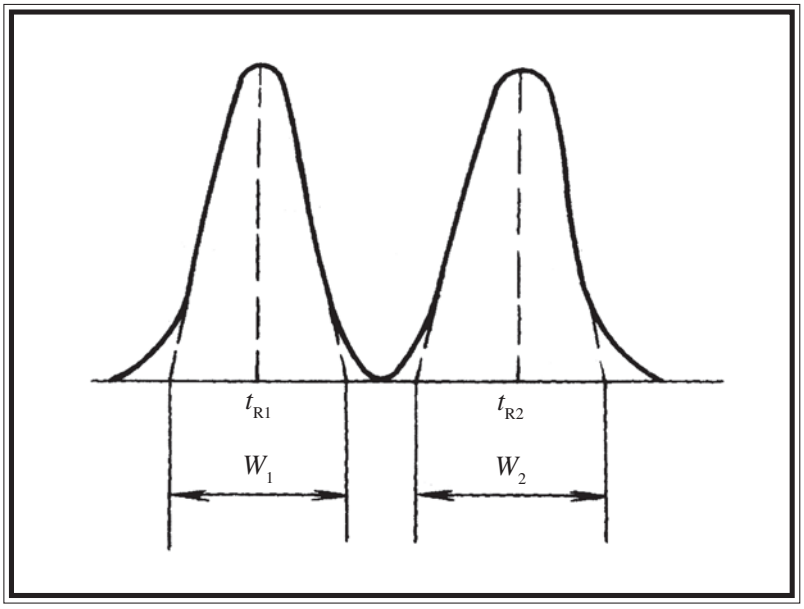


Figure 37 Resolution factor (*R*)

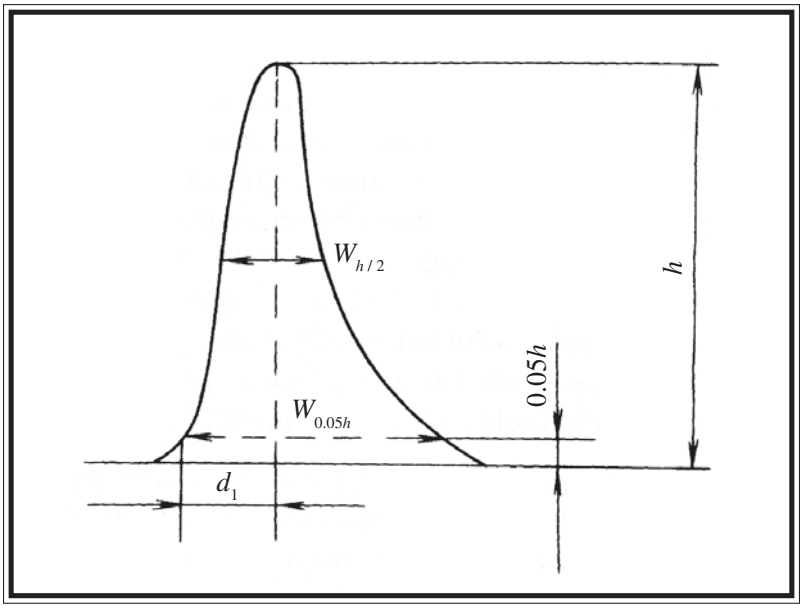


Figure 38 Tailing factor (*T*)

## Appendix IV(C): Chromatography – Gas Chromatography (GC)

GC is a separation technique consisting of gaseous mobile phase and a solid or immobilized liquid stationary phase. The sample is introduced through the sample injection port, heated and vaporized, and carried into the column by a carrier gas. Components of the test sample are separated in the column and pass through the detector in succession, a chromatogram is thus recorded.

(1) **General requirements of the apparatus** – Unless otherwise specified, nitrogen is employed as a carrier gas. A packed column or a capillary column may be employed for the test. A packed column is made of stainless steel or glass. The stationary phase of the column consists of active adsorbent, porous polymer beads or inert solid supports impregnated with liquid phase. A capillary column is made of glass or quartz with internal diameter of 0.2 or 0.32 mm. The stationary phase may be coated or chemically bonded to the inner surface of a column or supporting materials. The temperature of the sample injection port is usually set at 30–50 ˚C higher than that of the column itself. The volume of solution injected is usually no more than several micro-litres, the smaller the diameter of the column, the smaller the volume of solution is injected. Flame-ionization detector, electron-capture detector and mass spectrometric detector can be used to detect the separated components. The temperature of the detector is higher than that of the column itself, usually set at about 250–350 ˚C, but never below 100 ˚C. This will avoid condensation of the moisture.

Parameters including the type of the detector, stationary phase and the supporting material of the column 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. These include the internal diameter and the length of the column; the commercial brand and size of the supporting materials; the concentration of the liquid stationary phase; the flow rate of the carrier gas; the temperature of the column; the quantity of the injecting and the sensitivity of the detector, etc.

(2) **System suitability test** – The criteria for assessing the suitability of the system are the same as those set out in Appendix IV(B).

(3) **Procedure** – The procedure is the same as those set out in Appendix IV(B). Pay special attention to the effect of the change in room temperature and the injection time.