Chapter 2
Theoretical Basis of Thin Layer Chromatography (TLC)

2.1 Planar and Column Chromatography

In column chromatography a defined sample amount is injected into a flowing mobile phase. The mix of sample and mobile phase then migrates through the column. If the separation conditions are arranged such that the migration rate of the sample components is different then a separation is obtained. Often a target compound (analyte) has to be separated from all other compounds present in the sample, in which case it is merely sufficient to choose conditions where the analyte migration rate is different from all other compounds. In a properly selected system, all the compounds will leave the column one after the other and then move through the detector. Their signals, therefore, are registered in sequential order as a chromatogram. Column chromatographic methods always work in sequence. When the sample is injected, chromatographic separation occurs and is measured. This type of chromatography is known as “Online Chromatography”.

The different column chromatographic methods can be distinguished by their phase systems. Gas chromatography uses an inert gas such as nitrogen or helium as the mobile phase. In liquid chromatography the mobile phase is a liquid with a constant or varied composition altered during the separation process. A separation employing a constant mobile phase composition is known as an isocratic separation. If the mobile phase composition is varied during the separation this is called a gradient separation. A pump is used to move the mobile phase through the column at a suitable velocity. Separations are optimized by first selecting a suitable column and then varying the mobile phase composition to achieve the desired resolution in an acceptable time.

For planar separations like TLC, different samples are usually applied to the stationary phase before it is contacted by the mobile phase which begins to migrate through it in a definite direction. The movement of the mobile phase through the stationary phase is referred to as the development step. After development the mobile phase is removed by evaporation and detection is performed in the
stationary phase. The record of the detector response plotted against the separation distance is called a densitogram.

Separations by planar chromatography occur in parallel in contrast to the sequential approach of column chromatography. This situation has advantages and disadvantages: a sequential process like column chromatography facilitates automation in which a fixed protocol is commonly employed for a batch of samples.

Planar chromatographic separations are more flexible but not easily automated, and the sequence of manual steps commonly used makes validation of the method more difficult and has led, for instance, to the fact that the pharmaceutical industry hardly ever uses planar chromatography to check medicinal products (Fig. 2.1).

Another important difference between planar chromatography and column chromatography lies in a more flexible use of the stationary phase. A new stationary phase is needed for each separation in planar chromatography, thus preventing any cross-contamination from one sample to another. Thus even heavily contaminated samples can be applied to the stationary phase without sample cleanup. Sample components are not usually overlooked during detection because the whole separation can be scanned. Column chromatography only measures those substances that leave the

![Diagram of chromatography separation](image)

**Fig. 2.1** Separation of six dyes (CAMAG III mix, no. 032.8003), with the relevant densitogram, on a SiO$_2$ plate, developed with toluene. With increasing $R_f$ values: Ciba F-II (violet), indophenol (indigo blue), ariabel red (red), sudan blue II (blue), sudan IV (scarlet red) and (6) $N,N$-dimethylaminobenzene (yellow)
column. Those that remain on the column may be easily overlooked. It is also difficult to observe decomposition on the column during the separation process.

A significant difference between the two separation methods lies in the method of detection. In column chromatography, the sample is determined in the mobile phase and this restricts the number of useable liquids. Liquid chromatography is dominated by reversed-phase separations in which 90% or so of the separations employ a mobile phase consisting of acetonitrile or methanol in water or an aqueous buffer. In TLC, the mobile phase is removed before detection, so it cannot interfere with the measurements. On the other hand, detection is now performed in the stationary phase, which is an opaque medium, leading to its own compromises with the manner in which detection is facilitated or hindered.

In principle, column and planar chromatographic processes represent different separation methods, each with its own strengths and weaknesses. Many people see non-existent rivalry between TLC and HPLC, but both methods can be applied as appropriate because they complement each other.

2.2 TLC Capillary Flow

The significant difference between HPLC and TLC lies in the way the mobile phase permeates the stationary phase. In HPLC, a pressure gradient imposed along the column is responsible for the flow of mobile phase, but in classical TLC the mobile phase moves through the layer by capillary forces. In TLC textbooks many variations of the classical TLC approach are described [1–11]. These include a whole series of processes in which the flow is forced through the layer and referred to collectively as forced-flow methods. If the mobile phase flow is maintained through the layer by placing an electric field across the layer the method is referred to as electro-planar chromatography (EPC). Analogous to HPLC the layer can be sealed at the normally open surface and pressure used to drive the mobile phase through the layer by a series of methods collectively referred to as overpressure layer chromatography or optimum performance laminar chromatography (OPLC). There are also additional methods like rotation planar chromatography (RPC), where the mobile phase flow is induced by centrifugal force. All the above-mentioned methods will not be further discussed here, as we will concentrate on classical TLC methods, where capillary forces control the flow of mobile phase.

In TLC, the porous stationary phase can be modelled as a bundle of extremely fine capillaries, whereby the mobile phase cohesion is distinctively superior to the capillary wall adhesion. The surface tension of the mobile phase is thus noticeably reduced, creating a pressure difference that propels the liquid through the capillaries. This type of mobile phase flow in TLC is known as capillary TLC, to distinguish it from forced-flow TLC.

The upward flow of the mobile phase comes to a standstill in a vertical chamber when the static counter-pressure caused by the rising fluid equals the surface tension forces. During development in a horizontal chamber, it is only the
increasing friction force that brings the capillary flow to a stop after a while. In the case of a dipped TLC plate, the position of the solvent front moves rapidly at first and then gradually slows down. The total distance \( Z_f \) that the front moves is a square root function of time:

\[
Z_f = \sqrt{\chi t}.
\]  

(2.1)

The proportionality factor \( \chi \) is known as the flow constant. This relationship expresses the fact that the capillary flow is not constant. This relationship is not valid when the mobile phase evaporates from the layer surface or condenses from the vapour phase. In developing chambers with a large gas volume, this is invariably the case. The adsorption and desorption of the mobile phase components by the layer then vary in a complicated way. To obtain reproducible mobile phase migration, it is important to use developing chambers with as little gas space as possible. In larger chambers, the evaporation of the mobile phase can be effectively suppressed by saturating the gas space with the mobile phase before developing the TLC plate.

The following expression for the flow constant takes into consideration both the internal friction caused by the capillary flow and the static counter-pressure from the rising mobile phase, but not vapour exchange \([6,11,12]\):

\[
\chi = 2k_0d_p \frac{\gamma}{\eta} \cos \vartheta
\]

(2.2)

with

\[
\begin{align*}
    k_0 & \quad \text{permeability constant} \\
    k_0 & = 6–8 \times 10^{-3} \\
    d_p & \quad \text{the average particle size of the stationary phase} \\
    \eta & \quad \text{viscosity of the mobile phase} \\
    \gamma & \quad \text{surface tension (permeability)} \\
    \cos \vartheta & \quad \text{cosine of the wetting angle} \ 
\end{align*}
\]

The higher the viscosity \( \eta \) and the lower the surface tension \( \gamma \) of the mobile phase, the slower the front will move (Fig. 2.2). The viscosity and surface tension quotient is referred to as the permeation factor. The permeation factor provides a standard measure of the mobile phase front velocity. The greater the permeation factor, the faster the mobile phase will flow through the layer. The permeation factor for di-isopropyl ether is 9.1, while for 1-propanol it is 1.05. Di-isopropyl ether migrates three times faster than 1-propanol:

\[
(Z_f)^2 = \left( 2k_0d_p \frac{\gamma}{\eta} \cos \vartheta \right)t. \tag{2.3}
\]
There is little difference in the time required for development between a horizontal and a vertical plate. If the development distance $Z_f$ is measured at various intervals and plotted as $(Z_f^2)$ against time, the results form a series of straight lines (Fig. 2.3).

For a low polarity mobile phase, the cosine of the contact angle is usually about one and can therefore be neglected in the above relationship. This is not the case for chemically bonded layers like RP-18 where the cosine of the contact angle can be reduced to zero in extreme cases. When the layer is no longer wet by the mobile phase, the relationship between the development distance and time is no longer linear.
phase, capillary forces are inadequate for flow, but this may be reversed if a surfactant is added to an aqueous mobile phase [6]. Equation (2.3) indicates a lower flow rate for layers prepared from smaller particles. The front moves a shorter distance per time unit. Development on HPTLC plates with an average particle size $d_p < 10 \mu m$ takes longer than for TLC plates with mean particle diameters of about 40 $\mu m$.

### 2.3 TLC Distribution Equilibrium

After the samples are applied to a TLC plate, the plate is placed in contact with the mobile phase and its development begins. During development, the substances applied to the plate are distributed between two distinct phases, the stationary and the mobile phases. The sample components interact with both the stationary and mobile phases according to whether the mechanism is dominated by adsorption or absorption process. In the first case the mechanism is called adsorption chromatography and in the second case partition chromatography.

#### 2.3.1 Adsorption Chromatography

Adsorption is a characteristic property of surfaces, particularly solid surfaces. Adsorption in TLC occurs at the surface of the particles of the stationary phase, which are in contact with the mobile phase. The forces involved in adsorption processes are van der Waal’s forces, dipole-type interactions, and complexation interactions like hydrogen bonding.

For chromatographic separations, the adsorption process must be reversible and only involves physical interactions. On inorganic oxide layers the more polar groups a compound has the stronger it is adsorbed. A compound’s structure and the system temperature also play a role in adsorption. Steric factors affect the extent of interactions with active sites on the surface of the layer and higher temperatures tend to weaken polar interactions in general due to the greater motion of the adsorbed species.

The balance of adsorption interactions at a constant temperature depends only on the solute concentration at the adsorbent surface and its concentration in the mobile phase. The ratio of the equilibrium concentration of a substance in the stationary and the mobile phases is the distribution coefficient $K$ for adsorption chromatography, also known as the repartition coefficient [6]:

$$K = \frac{c_S [g/g]}{c_m [g/cm^3]}.$$
where

\[ K \] repartition coefficient (dimension g/cm³)
\[ c_S \] substance concentration in the stationary phase
\[ c_m \] substance concentration in the mobile phase

The repartition coefficient has the dimension of cm³/g [6].

When the solute concentration in the sorbing phase logarithmically decreases with its concentration in the mobile phase, the situation is described as an isotherm according to Freundlich. If there is a linear relationship between the amount of adsorbed substance and the concentration in the liquid phase, and if it shows a saturation effect as all adsorption centres are covered, this is described as a Langmuir isotherm. Both processes result in concave adsorption isotherms.

It really does not matter what the relationship looks like, the important aspect is to work in the linear region of the isotherm, which will produce Gaussian-shaped peaks. Asymmetric zones will result at higher concentrations, in the range of the curved relationship. The stationary phase is overloaded here, so the mobile phase cannot bind more solute in spite of an increasing concentration. Non-adsorbed solute will migrate through the stationary phase faster than expected, a phenomenon known as “tailing” because the substance trails a line behind it like a tail (Fig. 2.4).

In the concave region of the adsorption isotherm, more of the substance is adsorbed by the stationary phase than in the linear region of the isotherm. This case is described as “fronting”. In adsorption chromatography, the stationary phase generally consists of silica gel, aluminium oxide, kieselguhr, or magnesium silicate.

![Fig. 2.4](image)

**Fig. 2.4** Representation of the relationship between spot shape and the sorption isotherm. (a) Gaussian profile, (b) fronting, and (c) tailing [9]
These substances are all polar and can easily store water in an activated condition. Such phases can be desiccated by heating, thus releasing and reactivating the active sites of the stationary phase. In return, water effectively de-activates the stationary phase. In adsorption chromatography, adsorption of water quickly leads to phase over load, explaining why a small amount of water can lead to a significant alteration in retention behaviour during adsorption chromatography.

2.3.2 Partition Chromatography

In the middle of the 1930s, Martin developed an apparatus for counter-current extraction, to separate substance with similar properties by distributing them between two immiscible solvents. Working with Synge in 1940, he noticed that the separation of mixtures could be carried out when merely one liquid was immobilized and the other flowed over it. Martin and Synge saturated silica gel with water and allowed chloroform to flow through this stationary phase. In this way, they were able to separate very similar substances from one another. Similar to silica gel, cellulose, kieselguhr, and aluminium oxide can adsorb water and thus act as the stationary phase in partition chromatography. The sample is distributed between water and the organic mobile phase according to Nernst’s distribution law. From this observation it is only a small step to paper chromatography, where the stationary phase consists of water immobilized by cellulose. The concept of paper chromatography was published by both researchers in 1943.

In partition chromatography, separation depends on the relative solubility of the sample components in two immiscible solvents brought into contact with each other according to the Nernst’s distribution law:

\[
K = \frac{c_S}{c_m} = \frac{V_m m_S}{V_S m_m}.
\]

Here,

- \( K \) partition coefficient (dimensionless)
- \( c_S \) substance concentration in the stationary phase
- \( c_m \) substance concentration in the mobile phase
- \( V_{S,m} \) volume of the stationary or mobile phase
- \( m_{S,m} \) substance mass in the stationary or mobile phase

Nernst’s law indicates that the quotient between the concentration of each substance in the mobile phase \( (c_m) \) and the stationary phase \( (c_s) \) at a given temperature is a constant, the so-called partition coefficient. The above-mentioned mass dependence of the partition coefficients is a result of the concentration definition:
Because a substance is distributed between the mobile and stationary phases, the substance amount $n$ can also stand for mass $m$. In reality, the partition coefficient $K$ is independent of the total substance concentration resulting in a linear isotherm. This type of distribution is called a Nernst function. However, if dissociation or association occurs in either phase, such as acid/base proton transfer or complex formation resulting in a convex or concave deviation from a Nernst distribution, then tailing or fronting zones are observed in planar partition chromatography. Thus the range from $K = 1$ to $K = 10$ is ideal for partition chromatography.

The dominant features in adsorption chromatography are the surface phenomena, and in partition chromatography the decisive role is played by the distribution between the two liquid phases. Therefore, in adsorption chromatography, the type, position, and number of the solute’s functional groups control the separation, while the solute’s overall polarity plays the same role in partition chromatography. However, there are no absolutely distinct differences between the two methods. For example, liquid-coated silica gel phases can have unoccupied adsorption sites, which can act as adsorption centres. In the so-called “end-capped” phases, adsorption-active centres are partially blocked by chemical reactions. For example, cellulose phases work by using surface-bound water as the stationary phase. If a dry cellulose layer is used for a separation, the cellulose primarily serves as an adsorption phase and thereby desiccates the mobile phase. Thus amino acids on a dry cellulose layer may be well separated in the first separation section but then “smeared” in the second. This is because the water required for the formation of the stationary phase is no longer available from the mobile phase. Chemically bonded aminopropyl phases behave like hydrophilic distribution phases. An acid mobile phase protonates the NH$_2$ groups, forming cationic centres. This modified stationary phase then acts as a surface-active ion exchanger.

Chemically bonded cyanopropyl layers show particularly ambivalent properties with respect to the retention mechanism. Separations by adsorption chromatography are observed with low polarity mobile phases. With mobile phases containing water the retention mechanism changes to reversed-phase partition chromatography. Due to this ambivalent behaviour, cyanopropyl layers are particularly appropriate for 2D separations (Fig. 2.5).

Whether partition or adsorption chromatography is involved in the final separation is of less importance. The vital question is whether the separation can be reliably reproduced. Densitograms with symmetrical peaks verify that the separation occurs in the linear region of the isotherm where acceptable reproducibility can be expected.
Thus, a distribution value can always be calculated in the case of symmetrical peaks; it is irrelevant whether this is attributed to partition or adsorption chromatography. The best way of defining this distribution value is via the mass of a substance found in the stationary phase \( m_s \) and the mobile phase \( m_m \):

\[
k = \frac{n_S}{n_m} = \frac{m_S}{m_m} = K \frac{V_S}{V_m}.
\]  

(2.4)

The distribution expression \( k \) is called the retention factor and is connected to the distribution coefficient via the phase ratio for the system.

2.4 The Retardation Factor \((R_f)\)

2.4.1 The Empirical \( R_f \) Factor

The \( R_f \) factor is used for the qualitative evaluation of a TLC separation. It is the quotient of the distance of the substance zone from the sample origin to the front of the mobile phase \( z_f \). Historically, Goppelsröder was the first person to use this \( R_f \) value (relation to front expression) to characterize planar separations:

\[
R_f \equiv \frac{z_s}{z_f - z_0},
\]  

(2.5)

Fig. 2.5 Separation behaviour of steroids on a chemically bonded cyanopropyl layer, under adsorption conditions (a) and reversed-phase partition conditions (b). The labelling of the x-axis refers to percent composition with acetone [Source: Merck, with permission.]
where

\[ z_s \quad \text{distance of the substance zone from the sample origin (mm)} \]
\[ z_f \quad \text{solvent front migration distance (mm)} \]
\[ z_0 \quad \text{distance between immersion line and sample origin (mm)} \]

By definition, the \( R_f \) value cannot exceed 1. To avoid the decimal point, the \( R_f \) value is sometimes multiplied by 100 and then described as the \( hR_f \) value. The value of the retardation factor in a given separation system at constant temperature depends entirely on the characteristic properties of the separated substances. It is important for identification purposes that \( R_f \) values are accurate and reproducible, but this is difficult to achieve, since it is almost impossible to adequately control all the experimental conditions that influence the separation process (Fig. 2.6).

This problem is avoided by defining a retardation factor for a standard substance (\( R_{st} \)) that has been already separated in the system:

\[ R_{st} = \frac{z_s}{z_{st}} \]

Here,

\[ z_s \quad \text{distance of the substance zone from the sample origin (mm)} \]
\[ z_{st} \quad \text{distance of the standard substance zone from the sample origin (mm)} \]

![Fig. 2.6 Calculation of \( R_f \) values](image-url)
2.4.2 The Thermodynamic $R'_f$ Factor

While only an empirical $R_f$ value can be provided from a densitogram, the thermodynamic $R'_f$ value (also known as the true $R_f$ value) correctly represents the behaviour of the substance in the separation system. The thermodynamic $R'_f$ value is defined as the fraction of time the analyte is dissolved in the mobile phase ($t_m$) in relation to the total development time ($t_{ms}$):

$$R'_f = \frac{t_m}{t_m + t_S}.$$

Here,

$t_m$ time analyte is dissolved in the mobile phase
$t_s$ time analyte is associated with the stationary phase

The relationship between the mass of the substance in the mobile and stationary phases is independent of time. Assuming a constant distribution between phases, the mass distribution for an analyte is described by

$$R'_f = \frac{m_m}{m_m + m_S},$$

where

$m_m$ sample mass in the mobile phase
$m_s$ sample mass in the stationary phase

Reverting to a concentration definition ($c=n/V=m/MV$), it follows that

$$R'_f = \frac{c_m V_m}{c_m V_m + c_S V_S} = \frac{V_m}{V_m + \frac{c_S}{c_m} V_S},$$

where

$c_{S,m}$ sample concentrations in the stationary or mobile phase (mol/L)
$V_{S,m}$ mobile or stationary phase volumes (L)

If the partition coefficient relationship is applied to this equation, the result is called the Martin–Synge equation:
Here,

\[ R_f' = \frac{1}{1 + K \frac{V_s}{V_m}} = \frac{1}{1 + k}. \quad (2.6) \]

\( K \) partition coefficient
\( k \) retention factor

The empirically measured \( R_f \) values are only identical with the thermodynamic \( R_f' \) values if one of the following conditions can be applied:

- If the phase ratio remains constant for the whole layer
- If the mobile phase composition does not change during development
- If the stationary phase is free of solvent prior to development
- If the solvent front velocity is the same as the mobile phase velocity at the spot position

But these conditions are never fulfilled in the real world. Therefore the observed \( R_f \) value will always be smaller than the “true” or thermodynamic \( R_f' \) value. \( R_f \) values ranging from 62 to 100% of the thermodynamic \( R_f' \) values are quoted in the relevant literature. A reasonable approximation for the thermodynamic \( R_f' \) value is obtained by multiplying the observed \( R_f \) value by 1.1. Moreover, here the \( R_f \) value is used without differentiating between the “measured” or “thermodynamic” values. If not mentioned further, the abbreviation \( R_f \) is taken to mean a correctly measured \( R_f \) value identical with the thermodynamic value.

### 2.5 Mobile Phase Composition

The mobile phase composition is rarely constant over the whole separation distance. Some deviations are nearly always observed near the sample origin and the solvent front. Therefore only use measured \( R_f \) values in the range 0.05–0.9 when selective solvation of the stationary phase leading to demixing is not a problem.

The observed distribution of the components of a ternary mobile phase mixture on a silica gel layer is shown in Fig. 2.7. The mobile phase consists of low polarity mesitylene and polar benzyl alcohol (mixed with ethyl acetate). At a 40 mm separation distance the mesitylene front gradient can be observed with a decrease in the concentration of benzyl alcohol. The explanation is simple. The relative concentration of mesitylene in the mobile phase is increased at the expense of the benzyl alcohol; the more polar benzyl alcohol is selectively adsorbed by active sites on the silica gel layer. Mesitylene, less strongly adsorbed by silica gel, is enriched in the mobile phase and forms a gradient at the solvent front. This illustrates an important feature of separations employing the development mode:
A solvent front composition gradient is formed in all types of development chromatography. The mobile phase composition is not identical with the solvent mixture chosen for separation. The composition gradient is formed during development, due to the mutual interactions between the mobile and stationary phases.

Figure 2.7 shows typical adsorption chromatography with normal phase development, that is, with a mobile phase composition that is less polar than the stationary phase. Substances of low polarity migrate within the low polarity solvent front gradient. Thus some separations can produce strong front signals caused by the low polarity sample components that migrate within the solvent front gradients. Figure 2.7 demonstrates that the benzyl alcohol concentration in the mobile phase drops off rapidly at a separation distance of 40 mm. The rather volatile mesitylene gathers at the front. In this system a constant mobile phase composition occurs only up to a separation distance of 40 mm. In this mobile phase $R_f$ values higher than 0.85 are meaningless since unseparated substances in this region move with the solvent front gradient.

It can also be deduced from Fig. 2.7 that all substances with $R_f$ values $< 0.85$ have been overrun by the low polarity gradient at the solvent front. Therefore all substance with a separation distance less than 40 mm have spent some time migrating in the solvent front gradients. This explains why the measured $R_f$ values differ from the “correct” thermodynamic $R_f'$ values.

Figure 2.8 illustrates a typical plot of the composition for a ternary mobile phase containing methanol in a normal-phase separation employing an octadecylsiloxane-bonded silica gel layer. The mobile phase consists of equal volumes of mesitylene and benzyl alcohol in methanol. In this case it is mesitylene, which has a higher affinity for the low polarity stationary phase RP-18 than benzyl alcohol and is selectively absorbed from the mobile phase by the layer. The main component of the mobile phase is the relatively polar methanol, which washes out the benzyl
alcohol resulting in a broad solvent front gradient. Under these conditions, polar substances remain unseparated within the front gradients. The small mesitylene signal in front of the benzyl alcohol peak indicates the evaporated mesitylene, which was adsorbed by the layer and is now absorbed by the moving solvent front. A change in the mobile phase composition can be seen below a separation distance of 5 mm. Here, the proportion of mesitylene in the mobile phase is less than in the original solvent mixture. Mesitylene is enriched in the first 5 mm of the stationary phase while the benzyl alcohol concentration is reduced. After a separation distance of about 5 mm the new equilibrium between phases forms a constant mobile phase composition. This explains why the $R_f$ values below 0.1 and above 0.85 should not be used for substance characterization.

2.6 Transfer of TLC Separations to Columns

The $R_f$ value characterizes the migration distance of the analyte for conditions pertaining to TLC. However, a substance can only move over the plate while dissolved in the mobile phase; otherwise it remains in place. If a substance has an $R_f$ value of 0.2, then it must have spent 1/5 of the development time in the mobile phase and 4/5 of the development time in the stationary phase. The value for the retention factor $k$ is thus calculated with $k = 4$. The above-mentioned relationship between the $R'_{f}$ value and the retention factor must be valid in order to arrive at the $R'_{f}$ value of 0.2:

$$R'_{f} = \frac{1}{k+1} = \frac{t_m}{t_m + t_S}.$$
The \( R'_f \) value is an analyte-specific constant for a given stationary and mobile phase combination. Thus, layer and column techniques have a common retention factor, as shown by the equation

\[
k = \frac{t_S}{t_m} = \frac{1 - R'_f}{R'_f}.
\] (2.7)

Transferring separation conditions from planar separations to HPLC has practical advantages since TLC separations are faster, in general, and less expensive than non-optimized separations by HPLC. The precondition for transferring retention data from TLC to column chromatography is that the distribution coefficient must be identical in both systems. This is the case, when using the same stationary and mobile phases. The Martin–Synge equation (2.6), set out according to the partition coefficient, yields

\[
K = \frac{1 - R'_f}{R'_f} \frac{V_m}{V_S}
\] (2.8)

If TLC and HPLC distribution coefficients are set as equal and if \( V_m \) represents the mobile phase volume and \( W \) the sorbent weight, the following relationship results between TLC and HPLC separations:

\[
\left\{ \frac{V_m}{W} \right\}_{\text{HPLC}}^{k} = \left\{ \frac{V_m}{W} \frac{1 - R'_f}{R'_f} \right\}_{\text{TLC}}.
\]

From the transformed Martin–Synge equation, it follows that the quotient of the \( R'_f \) values in the TLC equation is the same as the retention factor in HPLC. If divided by \( V_m/W \), it results in the following expression

\[
k_{\text{HPLC}} = \frac{\{V_m/W\}_{\text{TLC}}}{\{V_m/W\}_{\text{HPLC}}} \frac{1 - R'_f}{R'_f}.
\]

If the weight of the stationary phases and volumes of the mobile phases are known, the \( R'_f \) values determined by TLC can be used to calculate the retention factors expected for an HPLC separation. To avoid weight and volume determinations, the separation systems can be calibrated by experimentally establishing the relationship between the TLC and HPLC separations for a series of standards and using this information to predict HPLC separations for other substances once TLC data have been obtained [13].

### 2.7 The \( R_m \) Value

The thermodynamic \( R'_f \) value is not linearly related to the structural properties of a molecule. However, a linear correlation with structure exists if a logarithmic form of the \( R'_f \) value is used. This so-called \( R_m \) value was introduced in 1950 by Bate-Smith and Westall [14]:
2.8 Temperature Dependence of TLC Separations

The expression for the $R_m$ value when inserted in the Martin–Synge equation (2.8) yields

$$R_m = \lg \left( \frac{1}{R'_l} - 1 \right) = \lg (k). \quad (2.9)$$

By applying equation $\Delta \mu^0 = RT \ln K = 2.3RT \lg K$ for the chemical potential, it affords the important Martin relationship:

$$R_m = \lg \frac{V_S}{V_m} + \lg K. \quad (2.10)$$

$$R_m = \lg \frac{V_S}{V_m} + \frac{\Delta \mu^0}{2.3RT}. \quad (2.11)$$

The chemical potential $\Delta \mu^0$ describes the free energy change resulting from the transfer of a mole of analyte under standard conditions between phases. The Martin relationship is based on thermodynamic considerations and is valid for adsorption as well as partition chromatography. Martin explained the remarkable separating powers of chromatography by using this equation. If two molecules are only slightly different, for example, differ only in a single structural element, their difference in chemical potential is proportional to the structural element. This explains why large molecules with small structural differences can be separated. For separation, the individual structural differences are important, not the relative differences [7].

The Martin equation serves as a basis for quantitative structure–retention relationships. In the ideal case, the chemical potential of a substance is the sum of the partial contributions of its structural elements (atoms, functional groups, and bonds). Substance $R_m$ values can be calculated from homologous substances as a multiple of the basic structure, as illustrated in Fig. 2.9 for different aliphatic carboxylic acids. If a member’s $R_m$ values are known, then further $R_m$ values for additional members can be deduced.

2.8 Temperature Dependence of TLC Separations

The effect of temperature on thin layer separations is relatively weak in comparison with other influences. A change in temperature has a noticeable effect on the equilibrium composition of the mobile phase in contact with the stationary phase. Distribution constants are temperature dependent as are sorption isotherms. Higher temperatures favour evaporation of the more volatile components of the mobile phase into the gas phase as well as reducing the viscosity of the mobile phase. Furthermore, the temperature-dependent water content of the vapour phase plays an important role in adsorption chromatography. It is a well-known fact that TLC separations based on adsorption are generally stable and run well in temperate
climate zones but are a total disaster on hot summer days in the tropics. High air humidity obviously makes a difference and there is a temperature effect as well. The above temperature-dependent factors partially compete with each other, thus making it almost impossible to exactly predict the effect that changes in temperature have on a TLC separation. The Martin relationship combines temperature and $R_m$ values in a single equation where the $R_m$ value increases as the temperature decreases. Thus $R_f$ values should increase as the temperature declines at a constant absolute humidity for the gas phase \[6\]. At a constant relative humidity, the observed $R_f$ values rise with decreasing temperature. Thus, it can be concluded that the higher the temperature, the lower the stationary phase activity. Moreover, mobile phase viscosity declines at higher temperatures, thus increasing the velocity constant. This results in a higher separation of the zone centres but with increased spot diameters. Therefore, there is no substantive argument in favour of performing TLC separations at higher temperatures. On the other hand, some separations are only successful at low temperatures. For example, the separation of polycyclic aromatic hydrocarbon on caffeine-impregnated silica gel layers at $-20^\circ C$ \[16, 17\].

In summary, it can be stated that temperature changes in the region of $\pm 10^\circ C$ cause little to no alteration in the separation process, provided that the humidity of the system is kept constant. In practice, this means that a laboratory does need to maintain temperate temperatures to carry out TLC separations.

2.9 Advanced Theoretical Considerations

Chromatographic separations are determined by a combination of kinetic and thermodynamic properties. Thermodynamic properties are responsible for the retention behaviour and selectivity. Kinetic properties determine zone broadening during a separation \[18\].
The process of chromatographic separation can be compared to a liquid–liquid distribution in a separating funnel, except that the equilibrium between phases occurs several thousand times in a chromatographic separation. In addition, in a chromatographic separation the mobile phase passes through the stationary phase while the two phases are merely brought into contact and separated in a typical liquid–liquid distribution experiment. In column chromatography a substance is permanently distributed between both phases but only moves when in the mobile phase. A simulation of these processes, transferred to TLC, leads to the sample moving through the TLC layer in the form of a binominal distribution. Assuming that a certain amount of analyte \( n \) was applied to a TLC layer before contacting the mobile phase, the total substance amount is concentrated in a small zone and the mobile phase has not yet had any contact with the sample, this situation is represented by the equation

\[
\begin{pmatrix} 0 \\ n \end{pmatrix} = \begin{pmatrix} n_{\text{mobile phase}} \\ n_{\text{stationary phase}} \end{pmatrix} = n(g + \beta)^0.
\]

The nominator in this equation represents the mobile phase and the denominator the stationary phase. The meaning of \( g \) and \( \beta \) will be explained below. Equilibrium has yet to be established between the two phases, as all the sample \( n \) still lie in the starting zone of the stationary phase. After adding the mobile phase, some sample is distributed between the stationary and mobile phases, according to their individual retention factors. The factor \( n_S \) refers to the amount of substance that remains in the stationary phase and \( n_m \) to the amount dissolved in the mobile phase. At this moment the following expression is valid for the substance in the stationary phase:

\[ n_S = kn_m. \]

As the amount of sample is conserved, it must be distributed between both phases, and the following expression is also valid:

\[ n = n_S + n_m. \]

If the upper equation is substituted into the lower one, this leads to the expression

\[ n = n_m(k + 1) \]

and after rearrangement, it becomes

\[ n_m = \frac{1}{(k + 1)} n \equiv \beta n. \]

From the sample applied \( n \), the fraction \( \beta n \) will move into the mobile phase. The equation below represents the fraction of the amount of sample \( n \), which remains in the stationary phase:
\[ n_S = kn_m = \frac{k}{(k+1)} n \equiv \gamma n. \]

The fraction of immobilized sample that remains in the stationary phase is given by \( \gamma n \). The expressions for \( \beta \) and \( \gamma \) allow a shorter description of the separation processes. By definition, the fraction \( \beta \) changes phase while the fraction \( \gamma \) remains behind, thus leading to \( \beta + \gamma = 1 \).

In the next step, fresh mobile phase contacts the sample zone pushing the fraction \( n\beta \) of sample molecules onto a clean area of the plate. The fraction \( n\gamma \) of the sample is immobilized by the stationary phase and does not move. After the first equilibrium, the situation is represented as follows:

\[
\left\{ \frac{0}{n\gamma} \right\} + \frac{n\beta}{0} = \frac{n_{\text{mobile phase}}}{n_{\text{stationary phase}}} = n(\gamma + \beta)^1.
\]

The next equilibrium can be described as follows. The fraction of \( n \) in the mobile and stationary phases will be distributed according to their retention factors. From the substance amount remaining in the stationary phase, the fraction \( \beta \) of the amount \( n\gamma \) will move into the mobile phase (i.e. \( \beta n\gamma \)), while the fraction \( \gamma \) of \( n\gamma \) (i.e. \( \gamma n\gamma \)) remains at the sample origin in the stationary phase. From the substance amount \( n\beta \) in the mobile phase, the fraction \( \gamma \) will move into the stationary phase (i.e. \( \gamma n\beta \)), while the fraction \( \beta \) of \( n\beta \) remains in the mobile phase. If further clean mobile phase is introduced, then we have

\[
\left\{ \frac{0}{\gamma n\gamma} \right\} + \frac{\beta n\gamma}{\gamma n\beta} + \frac{\beta n\beta}{0} = \frac{n_{\text{mobile phase}}}{n_{\text{stationary phase}}} = n(\gamma + \beta)^2.
\]

If the individual fractions of \( n \) from the stationary and mobile phases are summed at the end of the three equilibrium stages described above, the results can be represented by a binominal expression. The exact form depends on the number of equilibrium stages. For \( x \) number of equilibria the result is represented by \( n(\beta + \gamma)^x \).

After many equilibria a useful picture of the distribution of a sample on the TLC layer results. For low retention factors \( (k \approx 1) \) and for an infinite number of equilibria, the binominal distribution merges into a Gaussian distribution of the type

\[
f(x) = n \frac{1}{\sqrt{2\pi x\gamma\beta}} e^{-\left(\frac{(x-x_f)^2}{2\gamma\beta}\right)}.
\]

It has often been said that substances move along the TLC plate as a sequence of Gaussian-shaped zones. This statement can only be regarded as approximate, since even the longest TLC plate lacks the capacity to allow for an infinite number of equilibrium stages. Therefore, TLC peaks recorded in a densitogram are correctly described as binominal functions. Nevertheless, the Gauss function still remains a
satisfactory approximation for many TLC separations using high performance layers. The distribution of the substance amount \( n \) as a Gaussian distribution, characterized by a mean value \( z_S \) and variance \( \sigma \), is defined as follows:

\[
f(x) = n \frac{1}{\sigma \sqrt{2\pi}} e^{-(x-z_S)^2/2\sigma^2}.
\]  (2.12)

For \( x = z_S \) the e-function is 1 and the Gaussian function approaches a maximum height of \( f(z_S) = (1/\sigma \sqrt{2\pi}) \times 1 \). The value \( z_S \) of a peak in a densitogram must be taken where the signal reaches its highest value. The width of a Gaussian distribution is defined as the distance between the median of the recorded peak and the inflection points. This width measurement is described as the standard deviation of the peaks. The square of the standard deviation is called the variance \((\sigma^2)\).

Figure 2.10 illustrates the distribution of a constant amount of caffeine at different migration distances. The caffeine was applied to the plate, recorded (narrow peak at the start), developed a short distance, and then recorded again. This process was repeated nine times. Note that the caffeine signal widens with each development. If the square of the separation distance \((z_S)\) is divided by the variance, this will at first result in a more or less constant value:

\[
\frac{z_S^2}{\sigma_S^2} = \frac{(x\gamma)^2}{x\gamma\beta} = \frac{x'\gamma}{\beta} = xk \equiv N'.
\]  (2.13)

The product of the retention factor \( k \) and the number of distribution steps provides a constant value \( x \) representing the efficiency as well as the migration.
distance in a TLC separation. This product of efficiency and migration distance is called the “real number of theoretical plates” and is represented by the symbol $N'$. If the relationship is formulated according to the standard deviation $\sigma$ of the peaks recorded in a densitogram, this results in the following equation:

$$\sigma_S = \frac{1}{\sqrt{N'}} z_S.$$  \hspace{1cm} (2.14)

Here,

- $\sigma_S$ standard deviation
- $z_S$ substance migration distance
- $N'$ real number of theoretical plates

This expression is generally valid and means that the peak width ($2\sigma_S$) increases with longer migration distances $z_S$, as is clearly shown in Fig. 2.10. Thus it follows that a chromatographic system can only separate a finite number of samples because an infinitely long separation distance would lead to infinitely broad peaks.

The expression “number of theoretical plates” or “plate number” in column chromatography refers to the column length corresponding to a single equilibration stage. The plate number in column chromatography can be calculated directly from a chromatogram because each substance, independent of its retention time, must migrate the same distance defined by the column length. In contrast, each separated substance in TLC has associated with it a different migration distance defined as a fraction of the solvent front migration distance. Therefore, in thin-layer chromatography, the maximum plate number $N$ determined at the solvent front position should be corrected for each substance in the chromatogram by the fraction of the solvent front migration distance they migrated using their $R_f$ values [6,11]:

$$N' = NR_f.$$  \hspace{1cm} (2.15)

If the value of the peak width at base for a Gaussian peak is used, with $w = 4\sigma$ (or more accurately, $w = 2 \times 1.96\sigma$), the following relationship is valid:

$$N' = \left(\frac{z_S}{\sigma_S}\right)^2 = 16 \left(\frac{z_S}{w_B}\right)^2,$$  \hspace{1cm} (2.16)

where

- $N'$ real plate number
- $z_S$ substance migration distance
- $w_B$ peak width at base $= 4\sigma$
N' describes the theoretical plate number for each substance corresponding to the fraction of the plate number for the solvent front migration distance that each substance migrates over.

The plate number at the solvent front migration distance \( N \) represents the maximum value possible for that separation. It is an inflated value because separations cannot be achieved at the solvent front at which \( N \) is calculated:

\[
N = \frac{1}{R_f} \left( \frac{z_S}{\sigma_S} \right)^2 = 16z_S \frac{(z_f - z_0)}{w_B^2}.
\] (2.17)

Here,

- \( N \) plate number at the solvent front migration distance
- \( \sigma_S \) standard deviation of the substance
- \( w_B \) peak width at base for the substance = \( 4\sigma_S \)

For those substances that reside at the sample application position there is no interaction with the mobile phase, and the migration distance is zero. For those substances that migrate at the solvent front there is no interaction with the stationary phase, and the number of equilibrium steps is therefore zero. In either case, this will make \( N \) or \( N' \) zero as well. The value for \( N \) is larger than zero only for substances with an \( R_f \) value in the range \( 0 < R_f < 1 \). A chromatographic separation can only take place when the separation performance of the system is other than zero.

Figure 2.11 shows a separation of six dyes, demonstrating the increase in peak width with increasing migration distance \( z_S \). A densitogram allows peak widths at base and migration distances to be extracted for calculation of plate numbers.

![Densitogram illustrating the separation of six dye substances measured at 252 nm (CAMAG dye mix no. III)](image)

**Fig. 2.11** Densitogram illustrating the separation of six dye substances measured at 252 nm (CAMAG dye mix no. III)
using (2.16) (see Fig. 2.12). Plate numbers up to 5,000 are achieved in TLC. Plate numbers up to 300,000 have been described for HPLC [6], although plate numbers < 25,000 are more typical for columns in general use.

The standard deviation of a peak is often determined by the peak width at half height rather than by the peak width at base. The height of a Gaussian peak is described by the fore factor of the e-function \( \frac{1}{\sqrt{2\pi}} \). The Gaussian function value at median peak height \( H_{P/2} \) is given by

\[
\frac{H_P}{2} = \frac{1}{2\sigma\sqrt{2\pi}} = \frac{1}{\sigma\sqrt{2\pi}} e^{-\left(\frac{x}{\sigma}\right)^2/2\sigma^2}.
\]

From which it can be concluded that

\[
2 = e^{\left(\frac{x}{\sigma}\right)^2/2\sigma^2} \quad \text{and} \quad \ln 2 = \frac{\left(\frac{x}{\sigma}\right)^2}{2\sigma^2}.
\]

The peak width at half height \( w_H \) runs from \(-x\) to \(+x\). Thus

\[
w_H^2 = 8\sigma^2 \ln 2 = 5.545\sigma^2. \quad (2.18)
\]

The relationship for the plate number can also be written in the following form when the peak width at half height is used as a surrogate determination of the standard deviation for a Gaussian peak recorded in a densitogram:

\[
N = 5.545\sigma \frac{z_f - z_0}{w_H^2}. \quad (2.19)
\]
How can a separation be improved? Of course you would like to separate a substance from all the other sample components so that it can be correctly quantified. For this purpose a chromatographic system must be selected that provides sufficient differentiation of the $R_f$ values of the individual substances. At the completion of the development step the substance zones occupy the space defined by the solvent front migration distance characterized by a location ($R_f$ value) and a distribution that can be approximately represented by a Gaussian function. Because of dispersion, individual zones occupy space on the layer that depends on their $R_f$ value and system properties.

The peak area of the Gaussian distribution is proportional to the amount of substance contained in the spot. The peak width at base ($w_B$) of a Gaussian peak is a measure of the space occupied by the scanned zone on the TLC plate. This can be calculated for an individual substance by using the distance $z_S$ according to

$$w_B = \frac{4}{\sqrt{\pi N R_f}} z_S. \quad (2.20)$$

The resolution, $R_S$, of two neighbouring Gaussian curves (two peaks) is defined by the quotients from the difference between the two maximum signals ($z_{S1}$ and $z_{S2}$) and the arithmetic mean of their peak widths at base ($w_{B1}$ and $w_{B2}$):

$$R_S \equiv \frac{z_{S2} - z_{S1}}{w_{B1} + w_{B2}} = 2 \frac{z_{S2} - z_{S1}}{w_{B1} + w_{B2}} = \frac{z_{S2} - z_{S1}}{2(\sigma_1 + \sigma_2)}. \quad (2.21)$$

For $R_S = 0.5$ the distance between peaks is $\sigma_1 + \sigma_2 \approx 2\sigma$, called a “$2\sigma$ separation”. The two peaks still overlap each other by about 20%. However, the two components can still be recognized. At a resolution of 1, the peaks are almost completely separated. The peak profiles only overlap by 3%, corresponding to a $4\sigma$ separation (Fig. 2.13).

A resolution of 1.25 is sufficient for quantitative measurements by scanning densitometry. A resolution greater than 1.5 is unnecessary for quantifying overlapping peaks since the overlap of the peaks is less than 0.3%. Of course, this is only true for symmetrical peaks adhering to a Guassian profile. In the case of fronting or tailing peaks, a $10\sigma$ separation is required for reliable quantification, which corresponds to a resolution of $R_S = 2.5$.

From the definition of the $R_f$ value, $z_S = R_f(z_f - z_0)$, and with the simplification $\sigma_1 \approx \sigma_2 \approx \sigma$ the expression for the resolution can be transformed into

$$R_S = \frac{(R_{f2} - R_{f1})(z_f - z_0)}{4\sigma}. \quad (2.22)$$

With $(z_f - z_0) = z_{S1}/R_{f1}$ and application of (2.14), it follows that
The value of $R_f$ is calculated from the mean value of $R_{f1}$ and $R_{f2}$. Given that $R_f = 1/(1 + k)$ it follows that

$$R_S = \frac{(R_{f2} - R_{f1})}{R_{f1}} \frac{z_{S1}}{4\sigma} = \frac{R_{f2}}{R_{f1}} - 1 \left(\frac{1}{4}\sqrt{NR_f}\right).$$

Using the expression for the retention factor of the second substance $k_2 = (1 - R_{f2})/R_{f2}$, Snyder’s equation for resolution in TLC is obtained [6, 19, 20]:

$$R_S = \frac{1}{4} \sqrt{NR_f} (k_1 - k_2) R_{f2} = \frac{1}{4} \sqrt{NR_f} \frac{(k_1 - k_2)}{k_2} k_2 R_{f2}.$$

According to Snyder’s equation, the resolution of two substances is influenced by three factors:

(a) The first term in the Snyder equation describes the layer quality. This is characterized by the plate number $NR_f$ and is dominated by the contribution of diffusion to zone broadening for well-prepared layers. Resolution can be improved by an increase in the plate number but only in proportion to the square root of $NR_f$. Increasing the $R_f$ value is predicted to increase the resolution of two closely migrating peaks but this is not the case since resolution goes through a maximum at around an $R_f$ value of 0.3. This is because of the opposing contributions of the first two terms in (2.21).

(b) The second term in (2.21) contradicts the sense of the first. The greater the $R_f$ value, the lower the resolution of two closely migrating zones. All substances
migrating with the solvent front have an \( R_f \) value of one and a resolution of \( R_S = 0 \). All substances that migrate in the region close to the solvent front have a limited number of interactions with the stationary phase and the probability of their separation is low.

(c) The selectivity term depends on the ratio of the retention factors. The greater the difference for the retention factors, the higher the chromatographic selectivity and the higher will be the resolution. The selectivity term is a measure of the ability of the separation system to distinguish between the two substances by their capability for different intermolecular interactions in the mobile and stationary phases.

Equation (2.21) can also be interpreted differently. The first two terms (a and b) describe the “potential resolution” of the TLC system. It is also a general measure of the locally variable separation performance of a chromatographic system at a particular migration distance. It can be used to calculate the actual resolution (\( R_S \)) of a pair of substances, by multiplying the terms a and b by the term c [6]. By adopting the abbreviation \( Q^2 = R_f(1 - R_f^2) \) both the terms a and b of (2.21) can be described as follows (with \( R_f \sim R_{f2} \)):

\[
NQ^2 = NR_f(1 - R_f^2)^2 = \left[ \sqrt[2]{NR_f(1 - R_{f2})} \right]^2.
\]

The product \( NQ^2 \) is proportional to the resolution squared and known as the “effective plate number”.

If \( R_{f2} \) is replaced by the retention factor, the above equation can be written as

\[
NQ^2 = NR_f \left( \frac{1 - \frac{1}{1 + k_2}}{1 + k_2} \right)^2 = N' \left( \frac{k_2}{1 + k_2} \right)^2.
\] (2.22)

The variation of the effective plate number with \( R_f \) values is evaluated graphically in Fig. 2.14.

![Fig. 2.14](image.png) Effective plate number, as a function of \( R_f \) values with \( N = 6,751 \), according to [21]
According to Fig. 2.14, the highest effective plate number is obtained with $R_f$ values of about 0.33. Satisfactory separations are only achieved in the $R_f$ region from 0.05 to about 0.9. For critical separations, the system should be adjusted so that critical pairs have an average $R_f$ value around 0.33. In TLC it is extremely difficult to improve the plate quality (represented by $\sqrt{N}$) by a factor of more than 2–3. However, the selectivity can be improved by a factor 10–50 through an intelligent choice of the mobile and stationary phases. In practice, it is usually more productive to optimize the mobile phase composition for a chosen stationary phase [6].

2.11 Zone Broadening in Planar Chromatography

We can distinguish between three different processes that contribute to zone broadening in TLC, commonly referred to as the $A$, $B$, or $C$ terms [6, 20, 22–30].

2.11.1 The $A$ term

The $A$ term is determined by the heterogeneity of the layer, which results from variations in the local packing density, the distribution of particle sizes and shapes, and the presence of additives in the layer such as binders and visualization indicators. Layer heterogeneity is responsible for flow heterogeneity. Flow is slower through the internal porosity system than through the interparticle spaces [6]. This effect is called Eddy diffusion and is directly proportional to the particle diameter. Guiochon and Siouffi were the first researchers to substitute the term Eddy diffusion (from the Giddings approach) for the considerably slower processes of liquid chromatography. They also used the dimensionless Knox constant to describe the packing quality of the layer [6]:

$$\sigma_{xA}^2 = A \frac{\sqrt{d_p^4}}{\sqrt{D_mE_Rt}}$$

- $d_p$ particle size
- $D_m$ diffusion coefficient in the mobile phase (cm$^2$/s)
- $t$ time spent in the mobile phase (s)
- $A$ Knox constant
2.11.2 The B term

Sample molecules in the mobile phase diffuse in all directions. According to Einstein’s law of diffusion, a substance zone broadens with time, as determined by its diffusion coefficient. Zone broadening in the mobile phase, expressed as variance $\sigma^2$, can be written as

$$\sigma^2 = 2D_m t,$$

where

$D_m$ diffusion coefficient in the mobile phase (cm$^2$/s)

$t$ time spent in the mobile phase (s)

The longitudinal zone broadening ($\sigma_x$), i.e. the zone spreading in the flow direction, is calculated from the zone broadening due to diffusion, corrected to account for the space occupied by the impenetrable sorbent particles, the labyrinth factor, and introducing the retardation factor to account for the fraction of the separation time the sample spends in the mobile phase:

$$\sigma_{xB}^2 = 2D_m \lambda_m R_f t = B D_m R_f t.$$

Here,

$\sigma_x$ longitudinal spot spreading

$\lambda_m$ labyrinth factor (mobile phase)

$t$ time spent in the mobile phase (s)

$B$ Knox constant

A similar situation is true for the transversal standard deviation $\sigma_v$ across the flow direction.

Partition chromatography requires a further contribution to account for longitudinal zone broadening [20, 22–27]. The fraction of sample in the solvated stationary phase with a diffusion coefficient ($D_s$) and labyrinth factor for of the stationary phase ($\lambda_s$) will slowly exchange with sample in the mobile phase resulting in additional longitudinal zone broadening expressed by [28]

$$\sigma_{xB}^2 = 2 \left( \lambda_m D_m + \frac{1 - R_f}{R_f} \lambda_s D_S \right) R_f t$$

$\sigma_x$ longitudinal spot spreading

$\lambda_m$ labyrinth factor (mobile phase)

$D_m$ molecule diffusion coefficient (mobile phase)

$\lambda_S$ labyrinth factor (stationary phase)

$D_S$ molecule diffusion coefficient (stationary phase)

$t$ time (s)
This relationship demonstrates that in partition TLC, zone broadening from diffusion results from contributions that occur in both the mobile and stationary phases. In the direction of development, zones broaden with an increase in $R_f$ values influenced by diffusion in the solvated stationary phase [6]. This results in the formation of elliptical zones. For smaller $R_f$ values there are few interchanges between the stationary and mobile phases and the zones remain round or compact. At higher $R_f$ values, the substance has few interchanges with the stationary phase and spends most of its time in the mobile phase, and thus hardly diffuses into the pores of the stationary phase. Zone broadening depends almost exclusively on diffusion in the mobile phase, with a sample zone forming a diffuse circle.

### 2.11.3 The C term

The $C$ term accounts for delays caused by mass transfer processes during sorption and desorption of solute molecules. It is inversely proportional to the separation time and the diffusion coefficient and proportional to the square of the particle diameter [26]:

$$\sigma_{xC}^2 = C \frac{d_p^2}{D_m R_f t}.$$  

Here,

- $\sigma_{xC}$: non-equilibrium process zone broadening
- $d_p$: particle size
- $D_m$: diffusion coefficient in mobile phase (cm$^2$/s)
- $t$: time (s)
- $C$: Knox constant

The sum of all the variations then describes the total variance of the zone broadening process:

$$\sigma_S^2 = \sigma_{xA}^2 + \sigma_{xB}^2 + \sigma_{xC}^2.$$  

### 2.11.4 Local Plate Height $H$

As already mentioned, $N$ (the plate number for the complete separation length) describes the separation capacity of a chromatographic system, i.e. the larger the $N$ is, the more substances can be separated. Instead of giving the plate number, the
local plate height $H$ is frequently given as a chromatographic separation characteristic. This is obtained by dividing the total separation distance by $N$ [6]:

$$H = \frac{z_f - z_0}{N} = R_f \sigma_S^2 \frac{z_f - z_0}{z_S^2} = \frac{\sigma_S^2}{z_S}. \quad (2.23)$$

The expression $H$ represents an (imaginary) fraction of the plate length over which, in theory, one equilibration step in the separation is achieved [6]. The “H value” is encapsulated in the acronym HETP (height equivalent to a theoretical plate).

### 2.11.5 The van Deemter Equation

The van Deemter equation describes the relationship between the local plate height $H$ and the individual factors that lead to zone broadening. This equation was originally developed for gas chromatography and later used in liquid chromatography. Guiochon and Siouffi published an adaptation for TLC [6, 28]. This equation illustrates the relationship between molecular diffusion, mass transport, and the local plate height $H$. It enables the optimum velocity to be forecast in column chromatography and the optimum separation distance in TLC. The equation also allows us to predict which particle and pore diameters afford optimum separation performance for the stationary phase. Therefore the modified van Deemter equation made a decisive contribution to the successful development of HPTLC layers.

The solvent front velocity, which corresponds to the local mobile phase velocity for the sample zone, is calculated according to the general definition of velocity:

$$u = \frac{z_f - z_0}{t}. \quad (2.25)$$

Given that $(z_f-z_0)=z_S/R_f$ the following is valid:

$$R_f t = R_f \frac{(z_f - z_0)}{u} = \frac{z_S}{u} \quad (2.26)$$

Divided by $z_S$, the sum of all variations can be described as a local plate height:

$$H = \frac{\sigma_S^2}{z_S} = \frac{\sigma_{xA}^2 + \sigma_{xB}^2 + \sigma_{xC}^2}{z_S}. \quad (2.27)$$

A modified van Deemter equation designed for adsorption chromatography can be written as follows [28]:

$$H = A d_p \left( \frac{d_p}{D_m} u \right)^{1/3} + B D_m \frac{D_m}{u} + C \frac{d_p^2}{D_m} u. \quad (2.28)$$
Here,

\[ D_m \text{ solute diffusion coefficient (mobile phase)} \]
\[ d_p \text{ average particle size} \]
\[ u \text{ mobile phase velocity}, u=(z_f-z_0)/t \]
\[ A–C \text{ Knox equation coefficients [29]} \]

Constant \( A \) characterizes the quality of the stationary phase, \( B \) the axial diffusion, and \( C \) the resistance to mass transport in the layer.

The value of \( H \) is mainly dependent on \( u \) (the mobile phase velocity). If the mobile phase moves slowly through the layer, diffusion dominates; broadening the separating zones resulting in a poor separation. If the mobile phase moves too quickly through the layer, equilibrium is not fully established and again a poor separation results. For any separation there is an optimum mobile phase velocity corresponding to a minimum value for \( H \). This optimum situation depends decisively on the particle size \( (d_p) \) of the layer.

According to Giddings [30], the first term (the \( A \) term) in the van Deemter equation describes the Eddy diffusion and mass transport in the mobile phase. This diffusion in all directions is due to various different local flow velocities in the stationary phase. The usual cause is varying particle geometry of the packing. The more uniform the packing of the stationary phase, the lower the value of constant \( A \).

A further contribution to eddy diffusion arises from the difference in local velocity within the layer. Velocity gradients exist within the interparticle channels, with a greater difference between flow velocity in the middle and at the sides of the larger channels than in narrower channels. Smaller particles promote smoother flow, so there is less diffusion. Of course, one cannot keep on reducing particle size, since the channels would get too narrow and be easily blocked.

The second term of the van Deemter equation (the \( B \) term) describes the effects of the mobile phase on molecular diffusion. This term has already been discussed in the section on “zone broadening in TLC”. The mobile phase velocity is inversely proportional to zone broadening. Consequently, the contribution of the \( B \) term to zone broadening decreases with increasing mobile phase velocity. In TLC, zone broadening is most noticeable for longer separation distances and at higher \( R_f \) values. This is a consequence of the use of capillary forces to promote and maintain the flow of mobile phase and is a considerable disadvantage for TLC compared with pneumatically regulated column systems.

The third expression in the modified van Deemter equation (\( C \) term) takes into consideration that adsorption and desorption of the sample from the stationary phase needs time. Some molecules are adsorbed and therefore fixed in position while others move forwards with the mobile phase, resulting in zone dispersion in the flow direction. As this effect is also connected with the packing surface, it directly depends on the squared particle diameter. Furthermore, it also takes into consideration that molecules in thin layers can move to the surface of the layer faster than those in thicker layers. A faster re-dissolution process also reduces dispersion [18].
2.12 Optimum Separation Conditions in TLC

In GC or HPLC an optimum mobile phase velocity is calculated as the minimum $H$ value from the van Deemter relationship. Unfortunately, as Geiss rightly points out [6], the mobile phase velocity for TLC is not constant and, as a consequence, the value of $H$ depends on the position of each zone in the chromatogram. Therefore stating an optimum mobile phase velocity is not relevant for TLC. However, the location dependence of the local plate height can be eliminated by creating a new value ($H/z_f$) for the local plate height. If this quotient for the local plate height is integrated for the space between the sample application point and the solvent front migration distance (from $z_0$ to $z_f$) an average plate height $H_M$ is obtained which is independent of the mobile phase velocity but not the flow velocity constant $\chi$. Geiss called this expression “the observed average plate height”, which is the sum of all local plate heights passed through during the development [6]. The TLC expression for the local van Deemter equation must be integrated over the whole separation distance [6, 28]:

$$H_M = \frac{1}{\int_{z_0}^{z_f} dz_f} \int_{z_0}^{z_f} H dz_f = \frac{1}{z_f - z_0} \int_{z_0}^{z_f} \left( \frac{d_p}{D_m} u \right)^{1/3} + \frac{BD_m}{u} + C \frac{d^2_f}{D_m} u dz_f.$$

By introducing the local flow velocity $u = dz_f/dt = \chi/2z_f$ (derived from the flow relationship $z_t^2 = \chi t$), this equation can be solved:

$$H_M = \frac{3}{2} A \left( \frac{d^4_p \chi}{2D_m} \right)^{1/3} \frac{z_f^2/3 - z_0^2/3}{z_f - z_0} + \frac{BD_m}{\chi} (z_f + z_0) + \frac{C \chi d^2_f}{2D_m(z_f - z_0)} \ln \frac{z_f}{z_0}. \quad (2.25)$$

The $A$ term and the $C$ term can be minimized by small $d_p$. The diffusion coefficient of the sample in the mobile phase ($D_m$) is difficult to optimize since it appears in the nominator of the second term as well as in the denominator of the first and third terms. The major contribution from diffusion appears in the $B$ term.

According to Einstein’s diffusion law, a zone boundary will expand in all direction in time $t$ by $2D_m t$. Consequently, separations should be reasonably fast to minimize band broadening. Unfortunately, the flow of the mobile phase is hindered by small particles in the stationary phase, which reduces the $A$ and $D$ terms and enlarges the $B$ term. Using a mobile phase with small diffusion constants will limit zone broadening as much as possible [6]. Small velocity constants also reduce the influence of the $B$ and $C$ terms. In addition, it should be noted that for $z_0$ values close to zero the logarithmic expression tends towards very large values. The distance between immersion line and sample application zone should not be too short. According to Saunders and Snyder [6, 31], the optimum relationship for $z_f$ to $z_0$ should lie between 7 and 33.

As demonstrated in (2.25) and Fig. 2.15, $H_M$ achieves a minimum value for a fixed development length. For a value of $z_0 = 1$ cm, the optimum development
length for a TLC separation is between 7 and 15 cm [18]. Thus Stahl’s standard values for classic TLC: \( z_0 = 1 \text{ cm} \) and \( z_f = 10 \text{ cm} \) [6] were extremely well chosen. As shown in Fig. 2.15, the optimum development length for HPTLC is about 4 cm [18]. The minimum plate height for capillary flow separations is always higher than for forced flow. HPTLC plates with smaller particles than TLC plates provide better separations, but a development distance of 5 cm should not be exceeded (Table 2.1) [33].

What conclusions can be drawn from (2.25)? The ideal situation is to maintain a constant flow for the whole separation distance, a condition which is only fulfilled in Optimum Performance Laminar Chromatography (OPLC). However, further details about this special method will not be discussed here.

An essential practical point is the importance of working with TLC plates with a small particle size distribution. This can be achieved by using high-performance thin layer plates. Moreover, the stationary phase should be homogenously packed,

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**Table 2.1** Typical TLC and HPTLC data, taken from [18]

<table>
<thead>
<tr>
<th>Parameter</th>
<th>TLC</th>
<th>HPTLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>(D_m)</td>
<td>(2.8 \times 10^{-5} \text{ cm}^2/\text{s})</td>
<td></td>
</tr>
<tr>
<td>(d_p)</td>
<td>8.8 (\mu\text{m})</td>
<td>6.0 (\mu\text{m})</td>
</tr>
<tr>
<td>Knox constants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(A)</td>
<td>2.83</td>
<td>0.75</td>
</tr>
<tr>
<td>(B)</td>
<td>1.18</td>
<td>1.56</td>
</tr>
<tr>
<td>(C)</td>
<td>0.84</td>
<td>1.42</td>
</tr>
<tr>
<td>Flow constant</td>
<td>(0.044 \text{ cm}^2/\text{s})</td>
<td>(0.019 \text{ cm}^2/\text{s})</td>
</tr>
</tbody>
</table>

---

**Fig. 2.15** The variation of observed plate height as a function of solvent front migration distance for conventional (TLC) and high-performance (HPTLC) silica gel layers, under capillary flow and forced flow conditions (Taken from [33] with permission. © Elsevier.)
which argues against preparing plates yourself and for purchasing industrially manufactured products.

If possible one should also use a solvent with low diffusivity in order to minimize zone broadening. In any case, development should always take place over the optimum separation distance [6]. Belenkii recommends using plates with $d_p = 10 \, \mu\text{m}$ and a development length of $z_f = 10 \, \text{cm}$ for substances of low molecular weight and layers with $d_p = 5 \, \mu\text{m}$ and a development length of $z_f = 5 \, \text{cm}$ for substances of higher molecular weight [12].

The $D$ term is minimized if the sample to be separated has a large stationary phase diffusion coefficient ($D_s$). This is more often the case in partition chromatography than in adsorption chromatography.

Van Deemter plots for layers prepared with particles smaller than $10 \, \mu\text{m}$ show constantly rising lines instead of the typical hyperbolic curves. Obviously, only the $B$ term (due to molecule diffusion in the mobile phase) contributes to peak broadening for these layers. All other contributions to band broadening can be neglected. Thus $H_M$ for small particle sizes ($d_p < 10 \, \mu\text{m}$) is reduced to the following [28]:

$$H_M = \frac{B}{\lambda} (z_f + z_0).$$

This equation demonstrates that a low plate height is achievable only for separations over a short distance. Therefore plates with larger particles must be used for separations over longer distances. In general, after choosing the plate material and optimum mobile phase, the analyst can only improve a separation by choosing an optimum development length.

2.13 Separation Number

A separation method that satisfactorily separates many substances must be rated higher than a separation system that can separate only a few substances. The separation number introduced by Kaiser provided the basis for an evaluation of the separation capacity of chromatographic systems [32]. The separation number describes the number of zones that can be separated with a resolution of $4\sigma$. In TLC this corresponds to the situation where the distance between two adjacent peaks in a densitogram is equal to the sum of their peak widths at half height. The peak width at half height $w_H$ can be expressed as a linear function of the development length. To calculate the separation number for TLC, Kaiser used an average peak width at half height value obtained by summing the peak width at half height for a sample zone at the origin and a sample zone at the solvent front obtained by extrapolation from a series of real peak widths recorded in a densitogram:

$$w_H = \frac{1}{2} (w_H(\text{start}) + w_H(\text{front})).$$
The average effective separation distance is calculated correspondingly as

\[ \bar{z}_M = \frac{1}{2}(z_f - z_0), \]

with the definition for the separation number (SN)

\[ SN = \frac{\bar{z}_M}{w_H} - 1 \]

or

\[ SN = \frac{(z_f - z_0)}{w_H^{\text{start}} + w_H^{\text{front}}} - 1. \] (2.26)

The separation number describes the number of separated zones over the separation distance \((z_f - z_0)\). To calculate separation numbers, the peak widths at half height can be determined according to the relation

\[ N' = \left( \frac{z_S}{\sigma_S} \right)^2 = 5.545 \frac{z_S^2}{w_{HS}^2}, \]

which proposes a linear relationship between the separation distance and the peak width at half height:

\[ w_H = w_H^{\text{start}} + w_{HS} = w_H^{\text{start}} + \sqrt{\frac{5.545}{N'_\text{real}}}z_S. \]

<table>
<thead>
<tr>
<th>Dye</th>
<th>Separation distance (mm)</th>
<th>( W_H ) (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciba F-II</td>
<td>0.92</td>
<td>0.73</td>
</tr>
<tr>
<td>Indophenol</td>
<td>2.57</td>
<td>0.83</td>
</tr>
<tr>
<td>Ariabel red</td>
<td>6.96</td>
<td>1.01</td>
</tr>
<tr>
<td>Sudan blue</td>
<td>9.53</td>
<td>1.10</td>
</tr>
<tr>
<td>Sudan IV</td>
<td>16.9</td>
<td>1.83</td>
</tr>
<tr>
<td>Dimethyl-aminobenzene</td>
<td>24.9</td>
<td>2.11</td>
</tr>
<tr>
<td>Front</td>
<td>34.8</td>
<td></td>
</tr>
</tbody>
</table>

As an example of the calculation, the separation of a dye mixture on silica gel (Fig. 2.11) provides the following values:

The slope of the plot of the peak width at half height against the separation distance yields a slope \( a = 0.0611 \) (Fig. 2.16). The intercept, calculated as peak
width at half height $w_{H(\text{start})}$, is 0.64 mm. The real plate number is calculated from the slope as

$$N'_{\text{real}} = \frac{5.545}{a^2} = 5.545 \left( \frac{z_f - z_0}{w_{H(\text{Front})} - w_{H(\text{Start})}} \right)^2,$$

(2.27)

which takes into account zone broadening during the separation process.

$$N'_{\text{real}} = \frac{5.545}{a^2} = \frac{5.545}{0.06109^2} = 1,486$$

The peak width at half height for a compound migrating with the solvent front is calculated as

$$w_{H(\text{front})} = \sqrt{\frac{5.545}{N'_{\text{real}}}} (z_f - z_0).$$

With the experimental total separation distance of $(z_f - z_0) = 38$ mm it follows that

$$w_{H(\text{front})} = w_{H(\text{start})} + \sqrt{\frac{5.545}{1486}} 34.8 \text{ mm} = 2.77 \text{ mm}.$$
Combining (2.26) and (2.27) with $5.545 = 4\ln(4)$ provides an expression for the separation number [32]:

$$SN = \frac{1}{2} \sqrt{\frac{N'_{\text{real}}}{\ln(4)}} \frac{w_{H(\text{Front})} - w_{H(\text{Start})}}{w_{H(\text{Front})} + w_{H(\text{Start})}} - 1.$$  \hspace{1cm} (2.28)

For the dye mixture a separation number $SN = 9$ is obtained, a value that is fairly typical for TLC. Maximum separation numbers are achieved by making application zones as small as possible. With the chosen separation system, it is no longer possible to influence sample diffusion. However, spot geometry can be optimized by using appropriate equipment.

### 2.14 Real Plate Height

The theory of zone broadening is based on the unrealistic assumption that zone broadening only depends on processes that occur development. To account for the unavoidable zone broadening during sample application, the plate height must be corrected for zone broadening during sample application. This is described as a real plate height $H_{\text{real}}$. Kaiser suggested a simple and practical method for determining real plate heights [29–32]. The analyte peak width at half height ($w_{H(\text{HS})}$) is calculated as the sum of the application width at half height ($w_{H(\text{start})}$) and the signal width ($w_{H(\text{real})}$) caused by chromatographic development:

$$w_{H(\text{real})} = w_{H(\text{start})} + w_{H(\text{HS})}.$$

For the real plate height

$$H_{\text{real}} = \frac{z_f - z_0}{N'_{\text{real}}}$$ \hspace{1cm} (2.29)

and substituting for $N'_{\text{real}}$

$$H_{\text{real}} = \frac{(w_{H(\text{front})} - w_{H(\text{start})})^2}{5.545(z_f - z_0)}.$$ \hspace{1cm} (2.30)

In the case of the CAMAG dye mixture, the real plate height is calculated as $H_{\text{real}} = 23.5$ μm.

In order to compare TLC methods, the experimentally determined real plate heights should be quoted according to (2.30).
References

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