Chapter 2
Fundamentals and Theory of HPTLC-Based Separation

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Abstract High-performance thin-layer chromatography (HPTLC) is a form of thin-layer chromatography (TLC) that provides superior separation power using optimized coating material, novel procedures for mobile-phase feeding, layer conditioning, and improved sample application. It promotes for higher separation efficiencies, shorter analysis time, lower amounts of mobile phase, and efficient data acquisition and processing. The major parameters that influence separation of the constituents within a mixture are the partition coefficients, retention factor ($R_f$), and capacity factor of the individual constituents on the plate, selectivity of the mobile and stationary phase to the solutes, and the plate height that decide the separation efficiency as well as resolution of the individual constituents within a mixture. The partition coefficient is defined as the molar concentration of the analyte in the stationary phase to that in the mobile phase. $R_f$, a fundamental qualitative value, is expressed as the ratio of migration distances of an individual components of a mix relative to the mobile phase. Capacity factor ($k$) is a fundamental characteristic of a substance that determines its qualitative chromatographic behavior. It can be expressed as the ratio of the retention time of the substance in the stationary to that in the mobile phase and is influenced by the chemical nature of the two phases. The separation number (SN) that influences separation power of HPLC is defined as the highest possible number of components that are completely separated in a mixture under a gradient-free isocratic TLC. The efficacy of separation of two components of a mixture in a chromatogram is termed as resolution and is influenced by the selectivity of the components between the stationary and the mobile phase, mobile phase flow rate influenced by particle size and solvent strength that influence capacity factors.
Planar Chromatography

Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is a stationary phase over which the other mobile phase moves in a specific direction. Planar chromatography is a mode of chromatography in which the stationary phase is spread on a flat, planar surface. TLC is an important planar chromatographic technique that is widely used as a cost-effective method for rapid analysis of simple mixtures. It requires minimum sample cleanup as it uses a disposable stationary phase and has a high sample throughput because of its ability to analyze several samples in parallel. It allows greater flexibility and simplicity in sample evaluation because of the possibility of sequential detection by complementary techniques, postchromatographic derivatization for identification and quantification, archiving a separation for evaluation at a later time, and accessibility of the sample because of the planar format. It can be applied to samples where analyte lack a convenient chromophore making detection by other methods difficult and can be applied for analyzing samples with minimal prepurification. In some respects, the techniques of TLC and HPLC are complementary. Optimum resolution in TLC is obtained when \( R_f \) is about 0.25, and it is interesting to note that this corresponds to a retention factor of 3.0, which is in the optimal range found for column. Liquid chromatographic column methods provide better efficiencies, better mass-transfer properties and higher operating velocities. HPLC can also be fully automated making this method more popular for most analytical applications. By contrast, TLC is the most popular method because of its low cost, simplicity, and flexibility.

Theoretical Considerations

Separation Efficiency

In TLC, the flow of the mobile phase is not controlled as in the column methods. The position of the mobile phase at time \( t \) as it moves through a sorbent layer when governed by capillary forces is thus described by \( (Z_f)^2 = \kappa t \), where \( Z_f \) is the distance moved by the mobile phase from the sample origin and \( \kappa \) is the velocity constant. Velocity constant is dependent on external factors including saturation level of vapor phase in contact with the stationary phase. It is also related to the properties of the mobile and stationary phases by the equation

\[
\kappa = 2K_o d_p (\gamma/\eta) \cos \theta,
\]

where \( K_o \) is the permeability constant, \( d_p \) is the average particle diameter, \( \gamma \) is the surface tension of the mobile phase, \( \eta \) is the viscosity of the mobile phase, and \( \theta \) is the contact angle. Assuming a uniform particle size distribution, the velocity
constant increases linearly with the average particle size and thus the solvent front velocity is greater for coarse-particle layers. The velocity constant also depends linearly on the ratio of the surface tension of the solvent to its viscosity. Solvents that maximize this ratio are preferred for TLC. For silica gel layers, the contact angle for all common solvents is close to zero as all solvents wet it adequately. For reversed-phase layers containing bonded, long-chain alkyl groups the contact angle of the solvent increases rapidly with increasing water content of the mobile phase. At about 30–40% (v/v) water, \( \cos \theta \) becomes smaller than 0.2–0.3 (Wall 2005). The mobile phase is virtually unable to ascend the thin-layer plate, and chromatography becomes impossible. To improve solvent compatibility, modern reversed-phase TLC plates are prepared from large-size particles (10–15 \( \mu m \)) or from sorbents with a defined degree of modification that is lower than that of exhaustively silanized sorbents. Solvent compatibility is much less of a problem for polar, bonded sorbents such as 3-aminopropylsilanized and 3-cyanopropylsilanized sorbents, which are wetted by all solvents including pure water. Consequently, solvent with high viscosity and surface tension will migrate at much slower rate than that with low viscosity and surface tension. It is therefore advantageous to mix solvents with low \( \kappa \) values with those with higher values to improve migration rate, but care will need to be exercised to ensure that the solvents are miscible and that the appropriate polarity is maintained.

The mobile-phase velocity for the fine-particle layer declines rapidly with the migration distance until it becomes so slow that diffusion causes the spots to broaden. The coarse-particle layer is more permeable than the fine-particle layer, and both the solvent velocity and the efficiency are higher at longer plate lengths. For fine-particle layers with a development length of 5–7 cm, it should be possible to obtain up to about 5,000 theoretical plates, but it is nearly impossible to exceed this number using capillary flow-controlled development. It is futile to use solvent migration distances greater than this for HPTLC plates since the mobile-phase velocity declines to the point where zone broadening exceeds the rate of zone center separation. For coarse-particle layers (\( d_p = 15 \mu m \)), a development length of about 15 cm is required to obtain around 5,000 theoretical plates and, though higher numbers are possible, they will lead to long separation times. When the development length is optimized, the separation performance of conventional and high-performance TLC are not very different, the virtue of HPTLC being that it requires a shorter migration distance to achieve a given efficiency resulting in faster separations and more compact zones which are easier to detect by scanning densitometry (Fig. 2.1).

A popular approach for improving resolution under capillary flow-controlled conditions is to use multiple developments. Either one-dimensional or two-dimensional separations are possible in planar chromatography (Geiss 1972). Mobile-phase velocity can also be controlled by external means, such as in forced-flow development. The parameters of (2.1) above then become unimportant provided that sufficient pressure is available to provide the desired velocity. The limited range of mobile-phase velocities under capillary controlled flow prevent the optimum performance of a given stationary phase. This combined with shorter separation times is the rational theoretical reason to prefer forced-flow development in TLC.
The use of forced flow clearly lowers the plate height, especially for the HPTLC plates. In addition, analysis times can be decreased, and gradient elution can be used. We have thus seen how smaller particle sizes that are used in HPTLC affect separation efficiencies as a result of changes in solvent flow rate. We shall now discuss some of the major parameters that influence chromatographic system efficiency with special reference to TLC and in particular HPTLC.

**Partition Coefficient**

In a chromatographic system, the mobile phase passes over through the stationary phase. The components of the mixture ideally equilibrate or differentially partition
between the two phases. This results in differential rates of migration of the components of the mixture while passing through the system. Various components of the mixture are thus retarded in proportion to their interaction with the adsorbent. At any given time, a particular analyte molecule is either in the mobile phase, moving along at its velocity, or in the stationary phase and not moving at all in the downstream direction. The sorption–desorption process occurs many times as the molecule moves through the bed, and the time required to do so depends mainly on the proportion of time it is sorbed to the time it is held immobile. The ratio of the equilibrium concentration of an analyte in the stationary phase divided by its equilibrium concentration in the mobile phase is described by the distribution constant $K_a$, and is represented by the equation

$$K_a = \frac{C_S}{C_M}; \quad (2.2)$$

where $C_S$ is the concentration of the analyte in the stationary phase and $C_M$ is its concentration in the mobile phase. It is this ratio that controls the rate of migration of an analyte. As analyte proceeds through the system at a given temperature, it partitions itself between the two phases and is retained in the system in proportion to its affinity for the stationary phase. A solute with large $K_a$ has a great affinity for stationary phase and will travel slowly through the layer. Thus, for a mixture containing two constituents A and B, if A has a lower partition coefficient than B, the former will spend less time in the stationary phase and thus will migrate faster than the latter. For an ideal situation, the ratio of $C_S$ to $C_M$ will follow a linear correlation. However, if the spot/band is overloaded, the relationship of $C_S$ to $C_M$ becomes nonlinear. Overloading of sample usually results in a slight increase in $R_f$ value. This increases with loading concentration. Nonlinear curves are undesirable because they not only cause the $R_f$ value to vary but also can impair critical separations because of “tailing” effects and introduce unnecessary errors in quantification.

**Retention/Retardation Factor**

The position of any solute spot in TLC is characterized by its retention/retardation factor $R_f$. It is a fundamental qualitative value and is expressed as

$$R_f = \frac{\text{Distance travelled by the analyte} \ (Z_s)}{\text{distance travelled by the solvent front} \ (Z_f)}.$$

$R_f$ values range from 1.0 for analyte migrating to the solvent front to 0.0 for an analyte strongly retained at the point of application (Fig. 2.2).

It is sometimes more convenient to report as $R_f \times 100$. The reproducibility of $R_f$ values depends on many factors, such as quality of the sorbent, humidity, layer
thickness, development distance, and ambient temperature. Overloading of sample usually results in a slight increase in $R_f$ value. System errors affect this fundamental qualitative TLC value when the exact position of the solvent front cannot be located. In this case, the $R_f$ values become systematically too large. The loss of mobile phase or the “piling up” of mobile-phase components already present results in the values becoming too small. These $R_f$-values are not real and cannot be used to calculate $k$, the capacity factor. Real values can be obtained when no gradient exists along the separation path, no loss of mobile phase occurs, the correct position of the solvent front can be measured without errors, and by excluding any influences resulting from prevaporation. Consequently, a HPTLC chamber for obtaining real $R_f$ values has almost no gas phase, does not exhibit a temperature gradient, the layer is in a horizontal position, and is fully symmetrical. This is the only effective way of eliminating any disturbance of the phase ratios. In other approaches, complex equipment is used to compensate and regulate the many variables which influence the $R_f$ value. $R_f$, also known as the retardation factor in planar chromatography (such as TLC), has the same meaning, as the retardation factor $R$ in column techniques. A comparison of HPLC and TLC for systems with equivalent stationary and mobile phases can be made by comparing their respective $k$ values, each calculated with the appropriate retardation factor, $R$ or $R_f$. In conventional thin-layer chromatography, or especially for the techniques of overrun (continuous) or multiple developments, where the solvent front is not measurable, $R_x$ values can be recorded. $R_x$ is defined by the equation

$$R_x = \frac{\text{Distance traveled by solute}}{\text{distance traveled by standard substance}}$$

Unlike $R_f$, $R_x$ values can be greater than 1. Neither $R_f$ nor $R_x$ values are true constants, but $R_x$ values are more reproducible than absolute $R_f$ values and should be preferred for purposes of identification when comparing sample mobilities to tabulated values.

The optimum method for obtaining tentative identification of a substance is to spot the sample and a series of reference compounds on the same chromatogram. In this way, mobilities of all compounds are compared under the same experimental conditions, and a match in $R_f$ values between a sample and standard is evidence for
the identity of the sample. Experimental conditions should be chosen so that the compound to be identified moves to a point near the center of the layer ($R_f = 0.5$) and resolution between spotted standards is as good as possible. If $R_f$ values on silica gel are higher than desired, the polarity of the mobile phase is reduced. For $R_f$ values that are too low, the polar component of the mobile phase is increased. If the spots of interest from the sample do not correspond with the standards, they are either not the same or their mobilities have been affected by accompanying extraneous material from the sample. A correlation of the $R_f$ values between circular and linear HPTLC was proposed by Geiss and is expressed as $R_f\text{ linear} = (R_f\text{ circular})^2$. This relation was found to be completely valid when the starting point is exactly in the center of the circular chromatogram. If the substance is applied at a distance from the center point, the chromatogram will resemble a run in linear TLC.

**Capacity Factor**

The capacity factor of a substance is defined as “the ratio of its retention time in the stationary phase to that in the mobile phase.”

$$k = t_s / t_m.$$  \hspace{1cm} (2.3)

This is the simplest and most fundamental formulation of the qualitative chromatographic behavior of a substance that measures the degree of retention. A measurement of capacity factor will help to determine whether retention shifts are due to the stationary phase (capacity factor is changing with retention time changes) or due to the mobile phase (capacity factor remains constant with retention time changes).

$k$ is related to $R_f$ by the equation

$$R_f = 1/k + 1 \text{ or } k = (1 - R_f)/R_f,$$  \hspace{1cm} (2.4)

$k$ can also be described by the ratio of total number of moles of analytes in each phase. It is primarily controlled by the strength of the mobile phase, nature of the stationary phase, and the temperature at which separation is performed. If a substance does not migrate, i.e., not even a trace of the substance can be detected in the mobile phase, then its elution would last $\infty \times t_m$; the $R_f$-value is zero. A substance which migrates half of the separation length ($R_f = 0.5$) would require a time of $t_m + t_m$ for its elution. Its retention time in the mobile phase would be the same as the retention time in the stationary phase, and therefore $k = t_s / t_m = 1$. A substance which migrates with the front is not retained by the stationary phase. Its $R_f$-value is 1 and $k = t_s / t_m = 0/1 = 0$. When retention factor is less than one, elution of the analyte is so fast that accurate determination of its retention time is very difficult. High retention factors (greater than 20) mean that elution takes a very
long time. Ideally, the retention factor for an analyte is between 1 and 5. Thus, higher the capacity factor, the longer the retention time.

**Spot Capacity**

The separation number or spot capacity is defined as the maximum number of substances, which are completely separated between $R_f = 0$ and $R_f = 1$, provided that the separation conditions are isocratic. In quantitative chromatography, the separation number is defined as the highest possible number of completely separated substances, between $k = 0$ and $k = 10$. Two substances are completely separated, when the distance between the two adjacent peak maxima and the sum of both peak widths at half-peak height are same. It thus measures the efficiency of separation. Separation number is a quantity measurable with sufficient accuracy, simple, relatively independent of the chromatographic technique, easy to handle, and suitable for comparison and optimization.

Separation number is given by the expression

$$SN = \left( \frac{Z_f}{b_0 + b_1} \right) - 1.$$  

where

- $Z_f =$ migration distance of the front
- $b_0 =$ extrapolated width of the spot at $R_f = 0$
- $b_1 =$ extrapolated width of the spot with $R_f = 1$

In a TLC densitogram, widths are measured at half-height. A typical capillary-controlled HPTLC has a separation number of 10–20 while a capacity of 40–80 is possible for forced-flow TLC. A spot capacity of 100–400 and 500–3,000 is indicated for two-dimensional TLC with capillary flow and forced flow, respectively (Poole and Poole 1995). The accuracy of the $R_f$-value determination depends on SN and is interrelated by the formula

$$\Delta R_f = \frac{1}{SN + 1},$$

where $\Delta R_f = R_f$ differences between two adjacent, but completely separated spots.

**Plate Height**

The most common measure of the efficiency of a chromatographic system is the plate number, an analogy with distillation, originally called the number of theoretical plates contained in the chromatographic column system. Lower separation efficiency of TLC in comparison with liquid chromatography has resulted in the concept of theoretical plates being less frequently applied to this separation
technique. A successful attempt in recent years to enhance efficiency of the technique has, however, led to the application of theoretical plate concept to thin-layer chromatography. Broadening of a chromatographic spot can be simply expressed in terms of the theoretical plate number \( N \) of the given chromatographic system:

\[
N = 16 \frac{Z_f Z_s}{W_s},
\]

(2.7)

where, \( Z_f \) and \( Z_s \) are the migration lengths of the mobile phase and solute, respectively, and \( W_s \) is the chromatographic spot width in the direction of the mobile-phase migration.

\( N \) can also be calculated by the equation:

\[
N = 16 \left( \frac{Z_s}{W_s} \right)^2.
\]

(2.8)

The calculation of plate number based on this equation is demonstrated in Fig. 2.3.

Although the numerical values of \( N \) obtained for different solutes on the same chromatographic plate coincide fairly well, they usually differ significantly for another plate type. For this reason, the quantity \( N \) can be regarded as an approximate measure of the separating efficiency of chromatographic plates. It is proportional to the migration length of the mobile phase \( Z_f \), such that, when \( Z_s / W_s \) ratio remains constant, an increase in \( Z_f \) results in an increase of \( N \) and better separation. This proportionality of \( N \) and \( Z_f \) is given by the relationship

\[
N = \frac{Z_f}{H},
\]

(2.9)

where \( H \) is the so-called HETP value (i.e., height equivalent of a theoretical plate). The quantity \( H \), or simply the plate height, measures the efficiency of a given
chromatographic system per unit length of the migration distance, $Z_f$, of the mobile phase. Small $H$ values mean more efficient chromatographic systems and larger $N$ values. The main goal of efforts to enhance performance of thin layers is the attainment of small $H$ values and maximum $N$ values. As in other chromatographic techniques, the efficiency of a given TLC system is better (i.e., $H$ is smaller) for smaller particles of stationary phases or supports, lower mobile-phase flow rates, less-viscous mobile phases, and smaller solute molecules.

The plate height or number of plates is dependent on diffusion processes which result in a spreading of the sample spot. This is true for all chromatographic techniques. The determination of the number of plates is meaningful for the substance with $R_f = 1$, i.e., the substance which migrated the longest possible distance. One of the most important chromatographic relationships, the Van Demeter equation, attempts to estimate the relative contributions of eddy and molecular diffusion, and of the effects of mass transfer, on $H$. It is an empirical equation, originally established for column chromatographic techniques but valid also for thin-layer chromatography. The Van Demeter relationship can be written as

$$H = Aw^{0.33} + B/u + Cu + Du,$$  \hspace{1cm} (2.10)

where, $u$ is the flow rate of the mobile phase and $A$, $B$, $C$, and $D$ are the equation constants, measuring contributions of the different spot-broadening processes to the quantity $H$. The effects of eddy diffusion and mass transfer on the flowing mobile phase are described jointly by $A$. The molecular diffusion is reflected in $B$, while $C$ and $D$ correspond to the effects of mass transfer in the stagnant mobile and stationary phases, respectively. The constants $A$, $B$, $C$, and $D$ depend mostly on the parameters of the microporous solid, but they are also influenced by the nature of the solute and the mobile phase and by the working temperature of the chromatographic system.

**Resolution**

The main goal of chromatography is separation of constituents in a mixture. Chromatographic spots of two adjacent solutes may, however, overlap to a smaller or greater degree in many cases. The separation between two spots is measured by the quantity $R_s$, and is called resolution (Miller 2005). The $R_s$ of two adjacent chromatographic spots 1 and 2 is defined as being equal to the distance between the two spot centers divided by the mean spot widths (Fig. 2.4)

$$R_s = z_2 - z_1 / 0.5(w_1 + w_2).$$  \hspace{1cm} (2.11)

The quantity $R_s$ serves to define separation. When $R_s = 1$, the two spots are reasonably well separated. $R_s$ values larger than 1 indicates better separation while
smaller than 1 suggest a poor separation. Spot overlap becomes more disturbing when the concentration of solute in one spot is much greater than that in the other.

$R_s$ is also given by the equation

$$R_s = \frac{R_f(2) - R_f(1)}{0.5(w_1 + w_2)},$$  \hspace{1cm} (2.12)

where $R_f(1)$ and $R_f(2)$ are the $R_f$ values of chromatographic spots 1 and 2, respectively.

Assuming Gaussian concentration profiles of two closely spaced (i.e., overlapping) chromatographic spots and mean $R_f$ value for both of them ($R_f(1) \approx R_f(2) = R_f$), the above equation can be written as

$$R_s = 0.25 \left( \frac{K_2}{K_1} - 1 \right) \left( R_f N \right)^{1/2} \left( 1 - R_f \right),$$  \hspace{1cm} (2.13)

where $K_1$ and $K_2$ are distribution coefficients of solutes 1 and 2 between the stationary and mobile phases (“distribution” is used in a general sense and means partition, adsorption, or any other phenomenon, depending on the retention mechanism of a particular chromatographic technique). Equation (2.12) is the thin-layer chromatographic version of a fundamental chromatographic relationship that allows discussion of spot resolution in terms of the influence of $K_2/K_1$, $N$, and $R_f$. $K_2/K_1$ monitors interdependence between the stationary and mobile phases, $R_f$ monitors elution strength of the mobile phase, and $N$ depends on the length of the mobile-phase migration and on the plate height i.e., $Z_f$ and $H$, respectively.

**Selectivity**

Selectivity of separation is seldom referred to in the case of thin-layer chromatography, although no serious reason can be given for avoiding this term. To the contrary, selectivity of separation is a useful chromatographic notion, no matter
which particular technique, column or planar, is being considered. In the case of thin-layer chromatography, the separation factor $a$ can be defined as $a = K_2/K_1$ (14) which remains in full conformity with the definition used for the column technique. In fact, the quantity $a$ makes use of part of term I in (2.13) describing the resolution $R_s$ of two overlapping chromatographic spots. It can be stated that with greater difference between distribution coefficients of solutes 1 and 2 ($K_1$ and $K_2$), greater selectivity of separation ($a$) and better resolution ($R_s$) are observed. With $K_1 = K_2$, the two chromatographic spots entirely overlap ($a = 1$) and the respective spot resolution $R_s$ is nil. Several options for increasing $a$ are available, and these can be ranked in order of decreasing impact; change in mobile-phase composition, change in mobile-phase pH, change in stationary phase, change in temperature, and special chemical effects.

Notes

For the optimization of chromatographic separation, the conditions for separation on a specified coating material can be optimized by the right choice of mobile phase, i.e., its chemical composition. This can aid in achieving satisfactory separation within a minimum migration distance i.e., in shortest possible time. The minimum migration distance, necessary to obtain a separation (according to $R_f$-values) can be calculated when SN is known. The separation number, the result of a combined action between mobile phase and stationary phase, is determined by the plate height value. The plate height is dependent on the coating material and by starting spot width which in turn is a function of the sample loading (dosage) technique. A satisfactory resolution requires a minimum mobile-phase migration distance $Z_f$, i.e., a minimum analysis time and a good coating material (low plate height). Best results are obtained when the system has a high selectivity for the two substances to be separated or has a sufficiently high separation number and an optimum dosage technique. Combined with a maximum selectivity, it guarantees an excellent separation. Slight inadequacies in the sample loading can considerably reduce the quality of separation, even with excellent coating material. The effect of spread starting spot of a substance with a high $R_f$ value are less pronounced than those of a substance with low $R_f$ value. The experimental parameters discussed above are thus all important factors that should be optimized to achieve effective separation.

References


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