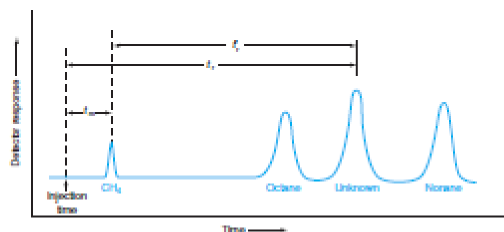


FIGURE 22-7 Schematic gas chromatogram showing measurement of retention times.

time. Figure 22-7 shows what might be observed when a mixture of octane, nonane, and an unknown are separated by gas chromatography, which is described in Chapter 23. The **retention time,** t_r, for each component is the time that elapses between injection of the mixture onto the column and the arrival of that component at the detector. **Retention volume,** V_r, is the volume of mobile phase required to elute a particular solute from the column.

Mobile phase or an unretained solute travels through the column in the minimum possible time, t_m. The **adjusted retention time,** t_r', for a retained solute is the additional time required to travel the length of the column, beyond that required by solvent:

Adjusted retention time:
$$t_r' = t_r - t_m \tag{22-14}$$

In gas chromatography, t_m is usually taken as the time needed for CH_4 to travel through the column (Figure 22-7).

For two components 1 and 2, the **relative retention,** α (also called *separation factor*), is the ratio of their adjusted retention times:

Relative retention:
$$\alpha = \frac{t_{r2}'}{t_{r1}'} \tag{22-15}$$

where $t_{r2}' > t_{r1}'$, so $\alpha > 1$. The greater the relative retention, the greater the separation between two components. Relative retention is fairly independent of flow rate and can therefore be used to help identify peaks when the flow rate changes.

For component 2 eluted after component 1, the *unadjusted relative retention*, γ, is the ratio of their unadjusted retention times:

Unadjusted relative retention:
$$\gamma = \frac{t_{r2}}{t_{r1}} \tag{22-16}$$

Unadjusted relative retention
$$= \frac{\text{retention time of component 2}}{\text{retention time of component 1}} = \frac{\text{speed of component 1}}{\text{speed of component 2}}$$

The unadjusted relative retention is the inverse of the ratio of the speeds at which the two components travel.

For each peak in the chromatogram, the **retention factor,** k, is the time required to elute that peak minus the time t_m required for mobile phase to pass through the column, expressed in multiples of t_m.

Retention factor:
$$k = \frac{t_r - t_m}{t_m} \tag{22-17}$$

Retention factor is also called *capacity factor, capacity ratio,* or *partition ratio* and was formerly written as k' instead of k.

The longer a component is retained by the column, the greater is the retention factor. It takes volume V_m to push solvent from the beginning of the column to the end of the column. If it takes an additional volume $3V_m$ to elute a solute, then the retention factor for that solute is 3.

EXAMPLE **Retention Parameters**

A mixture of benzene, toluene, and methane was injected into a gas chromatograph. Methane gave a sharp spike in 42 s, whereas benzene required 251 s and toluene was eluted in 333 s. Find the adjusted retention time and retention factor for each solute, the relative retention, and the unadjusted relative retention.

Solution The adjusted retention times are

$$\text{Benzene: } t_r' = t_r - t_m = 251 - 42 = 209 \text{ s} \qquad \text{Toluene: } t_r' = 333 - 42 = 291 \text{ s}$$

The retention factors are

$$\text{Benezene: } k = \frac{t_r - t_m}{t_m} = \frac{251 - 42}{42} = 5.0 \qquad \text{Toluene: } k = \frac{333 - 42}{42} = 6.9$$

The relative retention is expressed as a number greater than unity:

$$\alpha = \frac{t_r'(\text{toluene})}{t_r'(\text{benzene})} = \frac{333 - 42}{251 - 42} = 1.39$$

The unadjusted relative retention is

$$\gamma = \frac{t_r(\text{toluene})}{t_r(\text{benzene})} = \frac{333}{251} = 1.33$$

Test Yourself Ethylbenzene was eluted at 350 s. Find its retention factor and the relative retention and unadjusted relative retention for ethylbenzene and toluene. (*Answer:* 7.33, 1.058, 1.051)

Relation Between Retention Time and the Partition Coefficient

The retention factor in Equation 22-17 is equivalent to

$$k = \frac{\text{time solute spends in stationary phase}}{\text{time solute spends in mobile phase}} \qquad (22\text{-}18a)$$

Let's see why this is true. If the solute spends all its time in the mobile phase and none in the stationary phase, it would be eluted in time t_m. Putting $t_r = t_m$ into Equation 22-17 gives $k = 0$, because solute spends no time in the stationary phase. Suppose that solute spends equal time in the stationary and mobile phases. The retention time would then be $t_r = 2t_m$ and $k = (2t_m - t_m)/t_m = 1$. If solute spends three times as much time in the stationary phase as in the mobile phase, $t_r = 4t_m$ and $k = (4t_m - t_m)/t_m = 3$.

If solute spends three times as much time in the stationary phase as in the mobile phase, there will be three times as many moles of solute in the stationary phase as in the mobile phase at any time. The quotient in Equation 22-18a is equivalent to

$$\frac{\text{Time solute spends in stationary phase}}{\text{Time solute spends in mobile phase}} = \frac{\text{moles of solute in stationary phase}}{\text{moles of solute in mobile phase}}$$

$$k = \frac{c_s V_s}{c_m V_m} \qquad (22\text{-}18b)$$

where c_s is the concentration of solute in the stationary phase, V_s is the volume of the stationary phase, c_m is the concentration of solute in the mobile phase, and V_m is the volume of the mobile phase.

Partition coefficient $= K = \frac{c_s}{c_m}$

The quotient c_s/c_m is the ratio of concentrations of solute in the stationary and mobile phases. If the column is run slowly enough to be at equilibrium, the quotient c_s/c_m is the *partition coefficient*, K, introduced in connection with solvent extraction. Therefore, we cast Equation 22-18b in the form

Relation of retention time to partition coefficient:

$$k = K\frac{V_s}{V_m} \overset{\text{Eq. 22-17}}{=} \frac{t_r - t_m}{t_m} = \frac{t_r'}{t_m} \qquad (22\text{-}19)$$

which relates retention time to the partition coefficient and the volumes of stationary and mobile phases. Because $t_r' \propto k \propto K$, relative retention can also be expressed as

Physical basis of chromatography: The greater the ratio of partition coefficients between mobile and stationary phases, the greater the separation between two components of a mixture.

Relative retention:

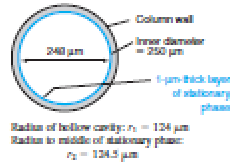
$$\alpha = \frac{t_{r2}'}{t_{r1}'} = \frac{k_2}{k_1} = \frac{K_2}{K_1} \qquad (22\text{-}20)$$

That is, the relative retention of two solutes is proportional to the ratio of their partition coefficients. This relation is the physical basis of chromatography.

EXAMPLE **Retention Time and Partition Coefficient**

In the preceding example, methane gave a sharp spike in 42 s, whereas benzene required 251 s. The open tubular chromatography column has an inner diameter of 250 μm and is coated on the inside with a layer of stationary phase 1.0 μm thick. Estimate the partition coefficient ($K = c_s/c_m$) for benzene between stationary and mobile phases and state what fraction of the time benzene spends in the mobile phase.

Solution We need to calculate the relative volumes of the stationary and mobile phases. The column is an open tube with a thin coating of stationary phase on the inside wall.



$$\text{Cross-sectional area of column} = \pi r_1^2 = \pi(124\ \mu\text{m})^2 = 4.83 \times 10^4\ \mu\text{m}^2$$

$$\text{Cross-sectional area of coating} \approx 2\pi r_2 \times \text{thickness} = 2\pi(124.5\ \mu\text{m})(1.0\ \mu\text{m}) = 7.8 \times 10^2\ \mu\text{m}^2$$

The relative volumes of the phases are proportional to the relative cross-sectional areas of the phases. Therefore, $V_s/V_m = (7.8 \times 10^2\ \mu\text{m}^2)/(4.83 \times 10^4\ \mu\text{m}^2) = 0.016\ 1$. In the preceding example, we found that the retention factor for benzene is

$$k = \frac{t_r - t_m}{t_m} = \frac{251 - 42}{42} = 5.0$$

Substituting this value into Equation 22-19 gives the partition coefficient:

$$k = K\frac{V_s}{V_m} \Rightarrow 5.0 = K(0.016\ 1) \Rightarrow K = 310$$

To find the fraction of time spent in the mobile phase, we use Equations 22-17 and 22-18a:

$$k = \frac{\text{time in stationary phase}}{\text{time in mobile phase}} = \frac{t_r - t_m}{t_m} = \frac{t_s}{t_m} \Rightarrow t_s = kt_m$$

where t_s is the time in the stationary phase. The fraction of time in the mobile phase is

$$\text{Fraction of time in mobile phase} = \frac{t_m}{t_s + t_m} = \frac{t_m}{kt_m + t_m} = \frac{1}{k + 1} = \frac{1}{5.0 + 1} = 0.17$$

Test Yourself Find the partition coefficient for toluene (t_r = 333 s) and state what fraction of the time it spends in the mobile phase. (***Answer:*** 430, 0.13)

FIGURE 22-8 Industrial-scale preparative chromatography column can purify a kilogram of material. The column volume is 300 L. [Courtesy Prochrom, Inc., Indianapolis, IN.]

Retention volume, V_r, is the volume of mobile phase required to elute a particular solute from the column:

Retention volume: $$V_r = t_r \cdot u_v \qquad (22\text{-}21)$$

where u_v is the volume flow rate (volume per unit time) of the mobile phase. The retention volume of a particular solute is constant over a range of flow rates.

Volume is proportional to time, so any ratio of times can be written as the corresponding ratio of volumes. If V_m is the elution volume for unretained solute,

$$k = \frac{t_r - t_m}{t_m} = \frac{V_r - V_m}{V_m}$$

where V_r is the retention volume for solute.

Scaling Up

We normally carry out chromatography for *analytical* purposes (to separate and identify or measure the components of a mixture) or for *preparative* purposes (to purify a significant quantity of a component of a mixture). Analytical chromatography is usually performed with thin columns that provide good separation. For preparative chromatography, we use fatter columns that can handle more load (Figure 22-8).[10] Preparative chromatography is especially important in the pharmaceutical industry, which can afford the high cost of separating compounds such as *optical isomers* of drugs (Box 23-1).

If you have developed a procedure to separate 2 mg of a mixture on a column with a diameter of 1.0 cm, what size column should you use to separate 20 mg of the mixture? The

most straightforward way to scale up is to maintain the same column length and to increase the cross-sectional area to maintain a constant ratio of sample mass to column volume. Cross-sectional area is πr^2, where r is the column radius, so the desired diameter is given by

Scaling rules:
- Keep column length constant
- Cross-sectional area of column $\propto$ mass of analyte:

$$\frac{\text{Mass}_2}{\text{Mass}_1} = \left(\frac{\text{radius}_2}{\text{radius}_1}\right)^2$$

(The symbol $\propto$ means "is proportional to.")
- Maintain constant linear flow rate:

$$\frac{\text{Volume flow}_2}{\text{Volume flow}_1} = \left(\frac{\text{radius}_2}{\text{radius}_1}\right)^2$$

- Sample volume applied to column $\propto$ mass of analyte
- If you change column length, mass of sample can be increased in proportion to total length

Scaling equation:

$$\frac{\text{Large mass}}{\text{Small mass}} = \left(\frac{\text{large column radius}}{\text{small column radius}}\right)^2 \tag{22-22}$$

$$\frac{20 \text{ mg}}{2 \text{ mg}} = \left(\frac{\text{large column radius}}{0.50 \text{ cm}}\right)^2$$

$$\text{Large column radius} = 1.58 \text{ cm}$$

A column with a diameter near 3 cm would be appropriate.

To reproduce the conditions of the smaller column in the larger column, the *linear flow rate* (not the volume flow rate) should be kept constant. Because the area (and hence volume) of the large column is 10 times greater than that of the small column in this example, the volume flow rate should be 10 times greater to maintain a constant linear flow rate. If the small column had a volume flow rate of 0.3 mL/min, the large column should be run at 3 mL/min.

The mass of sample (g) that can be run in preparative chromatography on a reversed-phase (page 603) silica-based column is *roughly*

$$\text{Column capacity (g)} \approx (2.2 \times 10^{-7}) L d_c^2 \sigma_g$$

where L is column length in mm, d_c is column diameter in mm, and σ_g is the surface area (m^2) per gram of stationary phase.[11] For $L = 250$ mm, $d_c = 50$ mm, and $\sigma_g = 200$ m^2/g, we estimate the column capacity as $(2.2 \times 10^{-7})(250)(50)^2(200) = 28$ g. This calculation presumes that the band will occupy the entire volume of the column, so it is surely an upper limit estimate. If you want the band to occupy just 20% of the column, so there is room for chromatography, the mass of sample would be $(0.2)(28 \text{ g}) = 5.6$ g.

22-4 Efficiency of Separation

Two factors contribute to how well compounds are separated by chromatography. One is the difference in elution times between peaks: The farther apart, the better their separation. The other factor is how broad the peaks are: the wider the peaks, the poorer their separation. This section discusses how we measure the efficiency of a separation.

Resolution

Solute moving through a chromatography column tends to spread into a Gaussian shape with standard deviation σ (Figure 22-9). The longer a solute resides in a column, the broader the band becomes. Common measures of breadth are (1) the width $w_{1/2}$ measured at a height

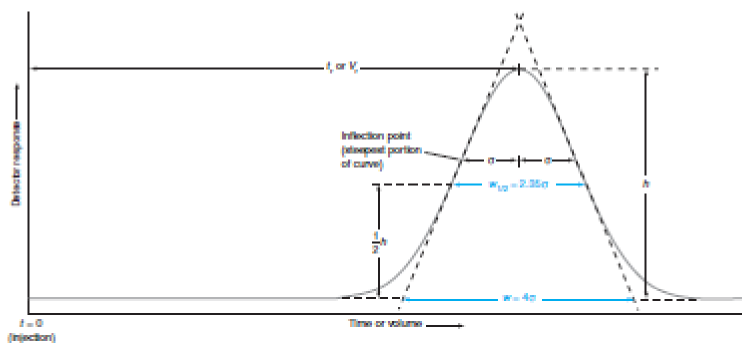


FIGURE 22-9 Idealized Gaussian chromatogram showing how w and $w_{1/2}$ are measured. The value of w is obtained by extrapolating the tangents to the inflection points down to the baseline.

rapid screening, e.g., in a synthetic organic chemistry laboratory to check if the desired compound is being made and how many impurities there are. The stationary phase is a thin layer of finely divided sorbent supported on a glass, metal (most commonly aluminum) or plastic sheet. Virtually any stationary phase used in HPLC can be used, provided a suitable binder can make it adhere well to the substrate. It differs with HPLC in that multiple samples can be simultaneously analyzed.

The three stages of injection, separation and detection in HPLC correspond in TLC to sample spotting, plate development, and detection (quite often just visual examination). In the simplest case, a pencil line is drawn horizontally towards the bottom of the plate (ca. 5–10 mm from the bottom). This is where one or more sample spots are applied. Samples (typically $0.5 - 5\ \mu$L) are spotted onto the line at regular intervals (ca. 20 mm apart) with a micropipette. The chromatogram is "developed" by placing the bottom of the plate or strip in the developing solvent (see margin figure). The solvent is drawn up the plate by capillary action, and the sample components move up the plate at different rates, depending on their relative affinities for the mobile vs. the stationary phase. Following development, the positions of the individual solute spots are noted. Different analytes move at a fraction of the rate of solvent movement; each analyte is thus characterized by the **R_f value**:

$$R_f = \frac{\text{vertical distance solute moves}}{\text{vertical distance solvent front moves}} \tag{21.23}$$

where the distances are measured from the original position of the sample spot and the solvent front is a line across the plate. In case an analyte spot tails or is diffuse, the position of maximum density is taken to be the post-development solute position. As with the retention factor in HPLC, the R_f value is characteristic for a given stationary phase–solvent combination. Few users or researchers today make their own TLC plates. Plate uniformity of commercial plates is generally good. However, when doing qualitative screening for a particular compound (or a set of compounds), it is always a good idea to spot the particular analytes on the same plate (singly or in a mixture) to compare the R_f values of the components in the test sample.

The principles of chromatography are illustrated by using TLC as an example in http://www.chemguide.co.uk/analysis/chromatography/thinlayer.html. This is a simple, short, and very readable account.

STATIONARY PHASES FOR TLC/HPTLC

Although essentially any HPLC stationary phase can be used in TLC, some 80% of TLC applications involve an unmodified 60 Å pore size silica stationary phase, according to Merck-Millipore, a leading vendor of TLC/HPTLC plates. Such an unmodified phase sees very limited use in modern HPLC. The remainder 20% comprises of CN-, diol-, $-NH_2$- or C18-modified silica and Al_2O_3, as well as cellulose. A typical TLC plate is 20×20 cm, although often narrower sections may be cut and used. Commonly, the sample can be applied on any edge of the plate and the plate can be accordingly developed with that edge at the bottom but some manufacturers also make plates with one area designated as the concentration zone. In such a case, the sample must be applied in this zone. Plates coded for good laboratory practice (GLP) also have specified direction of development. The mean particle size in TLC plates is 10–12 μm with a range of 5–20 μm (in HPTLC plates, mean size 5–6 μm with a range of 4–8 μm. The sorbent layer thickness of TLC plates is typically 250 μm on glass and 200 μm on other substrates, while that on HPTLC plates is 100–200 μm. Preparative plates have layer thicknesses of 0.5–2 mm and permit preparative separations in the gram scale. The typical migration distance on a TLC plate is 10–15 cm, while it is significantly smaller in HPTLC plates at 3–6 cm. However, the latter permits more efficient separations with typical plate heights of 12 μm (typical separation time 3–20 min) compared to 30 μm for a standard TLC plate (typical separation time 20–200 min). Although HPTLC plates are significantly more expensive, if a large

Merck-Millipore has an excellent instructional video about TLC and their TLC/HPTLC offerings at http://www.merckmillipore.com/chemicals/tlc-video/c_DIab.s1O39YAAAEqF1ck445w.

TLC (video) and HPTLC

number of analyses are to be conducted at a time, considering savings in time, the use of HPTLC plates may be more attractive. In addition, since band broadening is significantly less, nearly four times as many samples can be analyzed on an HPTLC plate than on a TLC plate and with better mass detection limits.

MOBILE PHASES FOR TLC

In adsorption chromatography, as with pure silica or alumina stationary phases, the eluting power of solvents increases in the order of their polarities (e.g., from hexane to acetone to alcohol to water). The developing solvent should contain no more than three components because mixed solvents tend to themselves migrate differently and themselves separate by chromatography as they move up the thin layer, causing a continual change in the solvent composition with distance on the plate. This may result in varying R_f values, depending on how far the spots are allowed to move up the plate. Minor variations in solvent composition and temporal/spatial variations in temperature may then cause major reproducibility problems in R_f. However, with appropriate controlled development chambers discussed later, reproducible solvent gradients may be deliberately exploited. The developing solvent must be of high purity. The presence of small amounts of water or other impurities can produce irreproducible chromatograms.

SAMPLE APPLICATION

Manual sample application with capillary micropipettes is usually performed for simple analyses. Sample volumes of 0.5 to 5 μL can be applied as spots onto conventional layers without intermediate drying. HPTLC plates can handle less sample; up to 1 μL per spot is acceptable. If samples are very dilute and more is needed for detection/visualization, the sample may be repeatedly applied on the same spot after drying.

More demanding qualitative, quantitative, and preparative analyses or separations are made possible only by automated instrumental sample application using the spray-on technique. To take full advantage of the separation power and reproducibility of HPTLC, precise automated positioning and volume dosage is mandatory.

At the high end, autosamplers/applicators are available, e.g., from CAMAG, that require no operator presence and can apply sample as spots by contact transfer or as a rectangular band by a spray-on technique, essentially using technology similar to that used in ink-jet printers. The spray-on technique permits sample application as bands or rectangles with volumes as little as 0.5 μL to $>$ 50 μL. Starting zones sprayed on as narrow bands offer the best separation attainable. Application in the form of rectangles also allows precise application of large volumes without damaging the layer. Prior to chromatography, these rectangles are focused into narrow bands with a solvent of high elution strength.

DEVELOPING THE CHROMATOGRAM

A TLC development chamber can be as simple as a beaker covered with a watch glass. Detailed considerations, however, reveal a much more complex system: a TLC system in fact is the only chromatographic system where a solid, liquid, and gas phase all play a role in the overall chromatographic process. While the role of the gas phase is typically ignored, it can significantly influence the result of the separation.

www.camag.com describes the many aspects of TLC and available equipment at different levels of sophistication to carry out the process.

The standard TLC chromatogram development involves placing the plate in a chamber, which contains a sufficient amount of developing solvent. The lower end of the plate is immersed several mm into the solvent, but not to the level of the sample application point. The solvent moves up the layer due to capillary forces until the desired distance is reached. The establishment of equilibrium between the component(s) of the developing solvent and the vapor phase is called chamber

saturation. The composition of the gas phase can differ significantly from that of the developing solvent and depends on the relative vapor pressures of the different solvent components. In adsorption chromatography, in a closed chamber, the following processes occur:

(a) The dry stationary phase adsorbs molecules from the gas phase. This adsorptive saturation processes also approaches an equilibrium: for silica and similar phases, in this process the more polar gas-phase components selectively transfer to the solid sorbent phase.

(b) Simultaneously the part of the sorbent already wetted by the mobile phase interacts with the gas phase. This results in selective transfer of the less polar components of the mobile phase to the gas phase. Unlike (a), this process is more governed by sorption equilibria than by vapor–liquid equilibria.

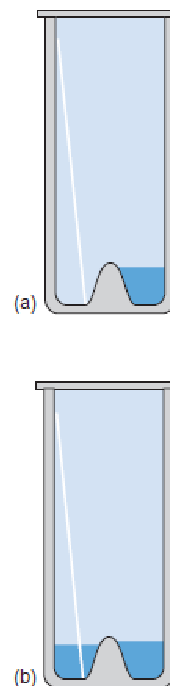
(c) During migration, the components of the mobile phase can themselves be potentially separated by the stationary phase, causing the formation of secondary fronts.

With the exception of a one component developing solvent, the terms "developing solvent" and "mobile phase," although used interchangeably, are really not the same. The mobile phase composition changes as it moves up the plate, while the liquid originally placed into the chamber is the developing solvent; only the composition of the latter is known. Processes (a) and (b) can both be experimentally manipulated by: putting developing solvent soaked material (e.g., paper towels) generously within the development chamber, waiting long enough for the developing solvent to saturate the chamber with vapors before beginning chromatography, and allowing the sample spotted plate to interact with the developing solvent vapors without contact with the liquid solvent (also called preconditioning). Processes (b) and (c), on the other hand, can be effectively stopped by placing a second TLC plate at a distance of one or a few mm facing the chromatographic layer of the analytical plate; this is called the "sandwich" mode of operation. The further an equilibrium according to (a) and/or (b) has been established and the less different the components of the mobile phase in their sorption behavior, the less important will be (c). In well-saturated chambers and with preconditioning, secondary fronts are rare. But secondary fronts are prominent in the sandwich mode.

With the exception of very polar components like water, methanol, etc., constituents of the developing solvent that are preferentially sorbed from gas phase may be pushed ahead of the real solvent front during chromatography. This results in R_f values being lower in saturated chambers and particularly on preconditioned layers, than in unsaturated chambers and sandwich configurations. Because of these possible complications from process (c), development in the sandwich mode or in an unsaturated horizontal developing chamber (see below) works best with single component solvents or mixtures where the behavior of the different components are very similar to each other.

It is important to understand that TLC development proceeds in the majority of cases under non-equilibrium conditions and all parameters must be maintained the same to obtain reproducible results. Development chambers are available in a number of different geometries. Chamber type and saturation play a dominant role; this also means that the precise R_f values may be different in each chamber even with the same developing solvents. No chamber is uniquely good but in some chambers, the relevant parameters can be better controlled.

The flat-bottom classical TLC development chamber shown previously in the margin figure is used under conditions of partial or complete saturation of the tank atmosphere with solvent vapors. The degree of sorbent presaturation cannot generally be controlled in such a chamber. In a twin-trough chamber, the floor of the classical chamber is modified with a partition of low height. Only one of the troughs may be filled with solvent to reduce solvent consumption. In addition, with one trough filled



A twin-trough chamber (a) top: used in the preconditioning mode, (b) bottom: development mode.