Part III gives some hints on what can be done to avoid errors. Again this synopsis is very heterogeneous in character. This does not diminish its value, of course.

Incompleteness is an inevitable feature of this book. I am grateful for all hints on other pitfalls and sources of error or on how to avoid them.

Recommended Texts

Veronika R. Meyer
Practical High Performance Liquid Chromatography
Wiley, Chichester
4th edition 2004

John W. Dolan and Lloyd R. Snyder
Troubleshooting LC Systems
Aster, Chester
1989

Paul C. Sadek
Troubleshooting HPLC Systems: A Bench Manual
Wiley-Interscience, New York
2000

Stavros Kromidas
Practical Problem Solving in HPLC
Wiley-VCH, Weinheim
2000

Stavros Kromidas
More Practical Problem Solving in HPLC
Wiley-VCH, Weinheim
2004

Lloyd R. Snyder, Joseph J. Kirkland and Joseph L. Glajch
Practical HPLC Method Development
Wiley-Interscience, New York
2nd edition 1997

Norman Dyson
Chromatographic Integration Methods
Royal Society of Chemistry, London
2nd edition 1998

Part I

Fundamentals

1.1 Chromatography

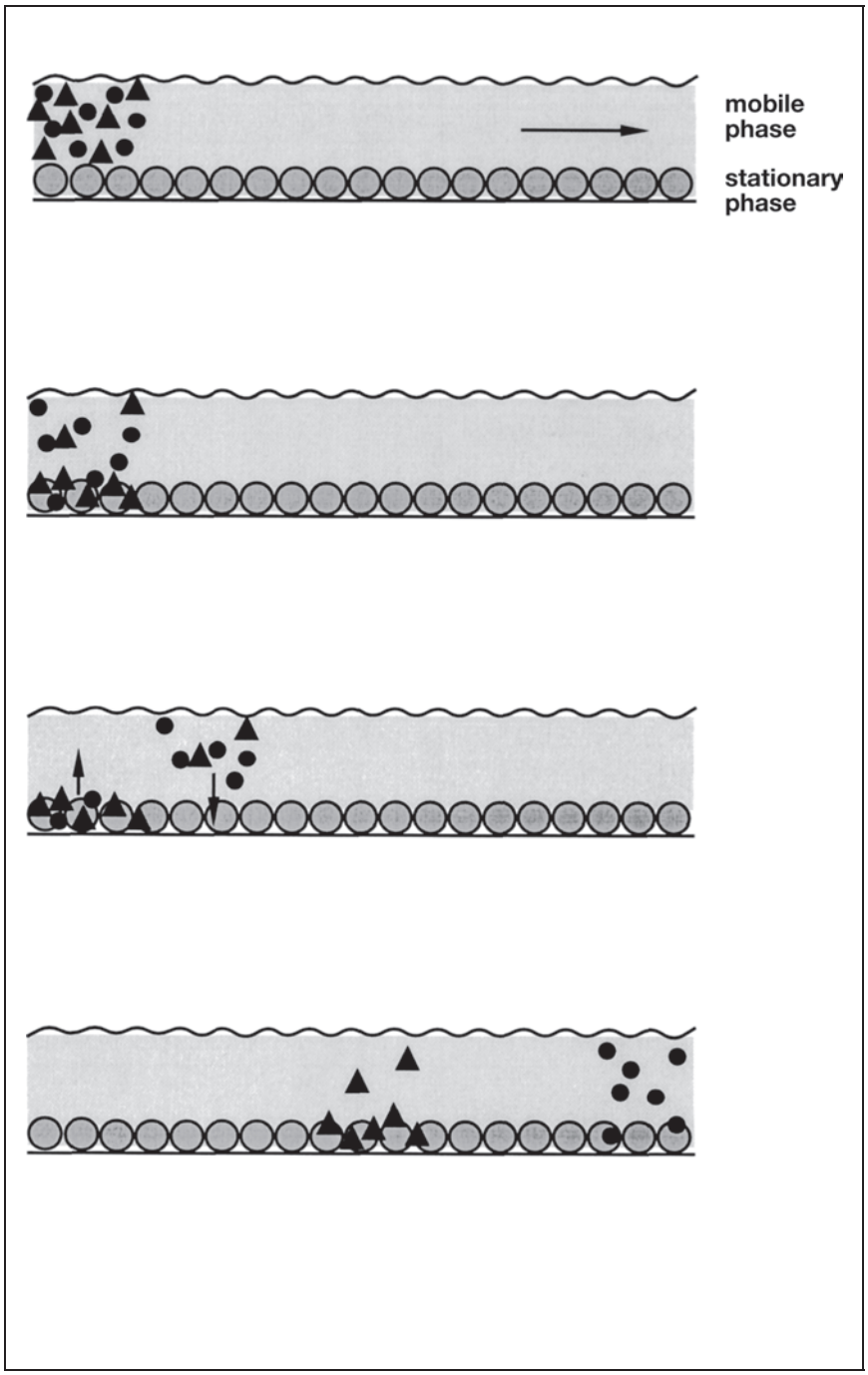
In chromatography, a physical separation method, the components of a mixture are partitioned between two phases. One of the phases stays in its place and is called the stationary phase, whereas the other moves in a definite direction and is called the mobile phase.

According to the type of mobile phase we distinguish between gas chromatography, supercritical fluid chromatography, and liquid chromatography.

The separation is based upon the different partition coefficients of the sample components between the two phases. It is helpful to divide the chromatographic column into small hypothetical units, the so-called theoretical plates. Within each plate a new partition equilibrium is established. The narrower a theoretical plate, the more equilibrium processes can take place within a column of given length and the more demanding the separation problems which can be solved.

The figure shows the separation of two compounds. One of these prefers the mobile phase but also enters the stationary phase. For the other compound the preference is the other way round. Thanks to this large difference in their properties the two types of molecule can easily be separated. They are transported through the column by the flow of the mobile phase and thereby reach zones where new equilibria are formed again and again.

In the drawing, such a theoretical plate has a height of approximately 3 1/2 stationary phase particle diameters. This height depends on the packing quality of the column, on the mass transfer properties of the phases, and on the sample compounds involved. Plate height is a function of the particle diameter of the stationary phase. For good columns, plate heights are equal to ca. 3 particle diameters irrespective of the particle size. A fine packing, e.g. with a 5-µm phase, gives four times as many theoretical plates as does a 20-µm packing if identical column lengths are compared. The column with the fine packing can therefore be used for more difficult separation problems.


mobile
phase
stationary
phase

1.2 Chromatographic Figures of Merit

To judge a chromatogram it is necessary to calculate some data which can be easily obtained. The integrator or data system yields the retention times, t_R, and peak widths, w; perhaps it is advisable to determine the peak width at half height, $w_{1/2}$. In addition the breakthrough time or 'dead time', t_0, must be known although it can be a problem to measure it unambiguously. In principle, the first baseline deviation after injection marks t_0. Then the following data can be calculated:

1. Retention factor, k (formerly capacity factor, k'):

$$k = \frac{t_R - t_0}{t_0}$$

The retention factor is a measure of the retention of a peak. It depends only on the phase system (the types of mobile and stationary phase) and on the temperature.

2. Separation factor, α:

$$\alpha = \frac{k_2}{k_1}$$

Two compounds can be separated only if α is higher than 1.0 in the selected phase system. For HPLC separations α should be 1.05 or higher (→ 1.3).

3. Theroetical plate number, N:

$$N = 16\left(\frac{t_R}{w}\right)^2 = 5.54\left(\frac{t_R}{w_{1/2}}\right)^2 = 2\pi\left(\frac{h_P t_R}{A_P}\right)^2$$

where h_P = peak height and A_P = peak area. The plate number is a measure of the separation performance of a column. (The equations given here are in principle only valid for symmetrical peaks.)

From the plate number it is possible to calculate the height, H, of a theoretical plate (e.g., in μm):

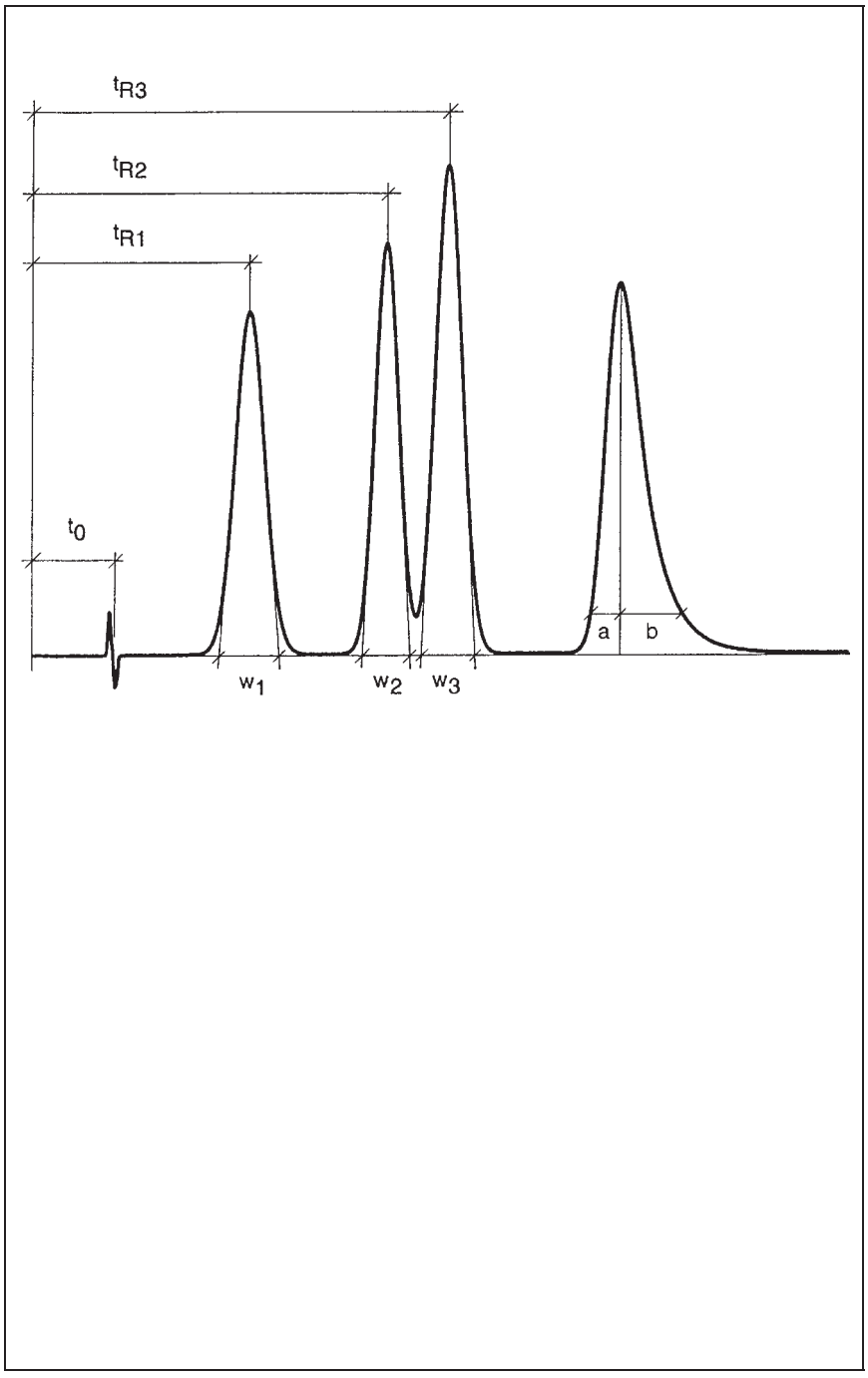
$$H = \frac{L_c}{N}$$

where L_c = column length.

4. Tailing T (for asymmetric peaks):

$$T = \frac{b}{a}$$

where a and b are determined at 10% of peak height.


t_{R3}
t_{R2}
t_{R1}
t_0
w_1
w_2
w_3
a
b

1.3 The Resolution of Two Peaks

The resolution of two adjacent peaks is defined as

$$R = 2\frac{t_{R2} - t_{R1}}{w_1 + w_2} = 1.18\frac{t_{R2} - t_{R1}}{w_{1/2_1} + w_{1/2_2}}$$

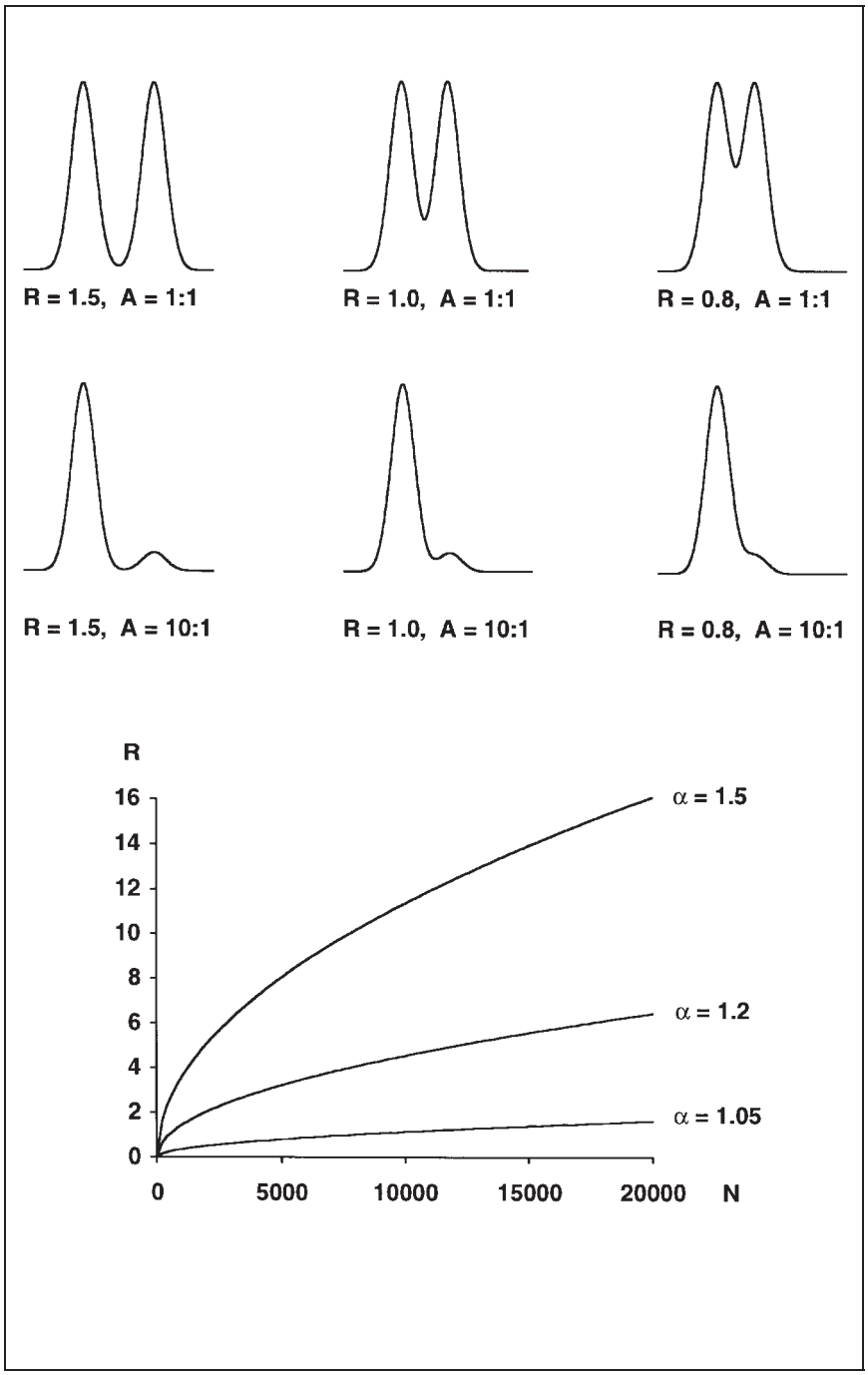
At a resolution of 1.0 the baseline between the peaks is not reached! Complete resolution is only obtained at $R = 1.5$ or higher, depending on the height ratio of the peaks. The smaller a peak compared with its large neighbor the greater is the resolution necessary to separate them.

The resolution depends on the separation factor α, the theoretical plate number N, and the retention factor k:

$$R = \frac{1}{4}(\alpha - 1)\sqrt{N}\frac{k}{1 + k}$$

This equation can be expressed in different forms, which are not of interest here. It is important to realize that the resolution is influenced by the three parameters. The separation factor has the largest effect. If a separation needs to be improved it is well worth the effort of increasing α, although it is impossible to give a general proposal concerning how to do this. If the plate number is increased, the effect is only by the factor $\sqrt{N}$; if the column length is, e.g., doubled, and by this also the plate number (at least in principle), the resolution will improve only by $\sqrt{2} = 1.4$. Increasing the retention factor only has a notable influence on resolution if k was small to start with.

The upper figure presents several pairs of peaks separated with varying resolution. The graph below demonstrates how the resolution increases with increasing plate number for three different separation factors.


R = 1.5, A = 1:1
R = 1.0, A = 1:1
R = 0.8, A = 1:1
R = 1.5, A = 10:1
R = 1.0, A = 10:1
R = 0.8, A = 10:1
R
16
14
12
10
8
6
4
2
0
0
5000
10000
15000
20000
N
α = 1.5
α = 1.2
α = 1.05

1.4 Reduced Parameters

The judgement and comparison of HPLC columns is best done with reduced, dimensionless parameters. A test chromatogram is acquired which enables the theoretical plate number, N, of the column to be determined from a suitable peak with low tailing. It is also necessary to measure the breakthrough time, t_0, with a refraction index peak or with an otherwise suitable compound (for reversed-phase separations, e.g., with thiourea). The pressure drop, Δp, under the given conditions is noted.

Then the following reduced parameters can be calculated:

1. Reduced plate height, h:

$$h = \frac{H}{d_p} = \frac{L_c}{N \cdot d_p}$$

h is a measure of the height of a theoretical plate as a multiple of the particle diameter, d_p. L_c is column length.

2. Reduced flow velocity, v:

$$v = \frac{u \cdot d_p}{D_m} = \frac{L_c \cdot d_p}{t_0 \cdot D_m}$$

v is a measure of the flow velocity in relation to the particle diameter, d_p, and the diffusion coefficient, D_m. In most cases D_m is not really known but it can be assumed to be $1 \cdot 10^{-9}$ m^2 s^{-1} for small molecules in water/acetonitrile and to $4 \cdot 10^{-9}$ m^2 s^{-1} for small molecules in hexane.

3. Reduced flow resistance, ϕ:

$$\phi = \frac{\Delta p \cdot d_p^2}{L_c \cdot \eta \cdot u} = \frac{\Delta p \cdot d_p^2 \cdot t_0}{L_c^2 \cdot \eta}$$

With ϕ the pressure drop can be described simply and clearly. It is, however, necessary to know the viscosity, η, of the mobile phase. Mixtures of water and organic solvents pass through a maximum of viscosity!

Favorable numbers: h = 3
v = 3
ϕ = 500 up to a maximum of 1000

$$h = \frac{H}{d_p} = \frac{L_c}{N \cdot d_p}$$

$$v = \frac{u \cdot d_p}{D_m} = \frac{L_c \cdot d_p}{t_0 \cdot D_m}$$

$$\phi = \frac{\Delta p \cdot d_p^2}{L_c \cdot \eta \cdot u} = \frac{\Delta p \cdot d_p^2 \cdot t_0}{L_c^2 \cdot \eta}$$

$h = 3$
$v = 3$
$\phi = 500$

D_m = diffusion coefficient [$m^2\ s^{-1}$]
d_p = particle diameter [m]
H = height of a theoretical plate [m]
L_c = column lenght [m]
N = theoretical plate number
Δp = pressure drop [$N\ m^{-2}$] (1 bar = 10^5 $N\ m^{-2}$)
t_0 = breakthrough time [s]
u = linear flow velocity [$m\ s^{-1}$]
η = viscosity [$Ns\ m^{-2}$] (1 mPas = 10^{-3} $Ns\ m^{-2}$)

1.5 The Van Deemter Curve

The separation performance of a column is not independent of the mobile phase flow rate. An optimum velocity, u_{opt}, is observed where the performance is highest. This relationship is described by the van Deemter curve which describes the height of a theoretical plate, H, as a function of the linear flow velocity, u. At u_{opt} the plate height, H_{min}, is smallest, which means that the number of theoretical plates, $N = L/H$, is largest. The peaks are narrowest and thus eluted with the largest possible height; the resolution reaches a maximum. Any deviation from the van Deemter optimum yields smaller peak heights and resolutions; yet the optimum velocity is not identical for all compounds of a sample mixture.

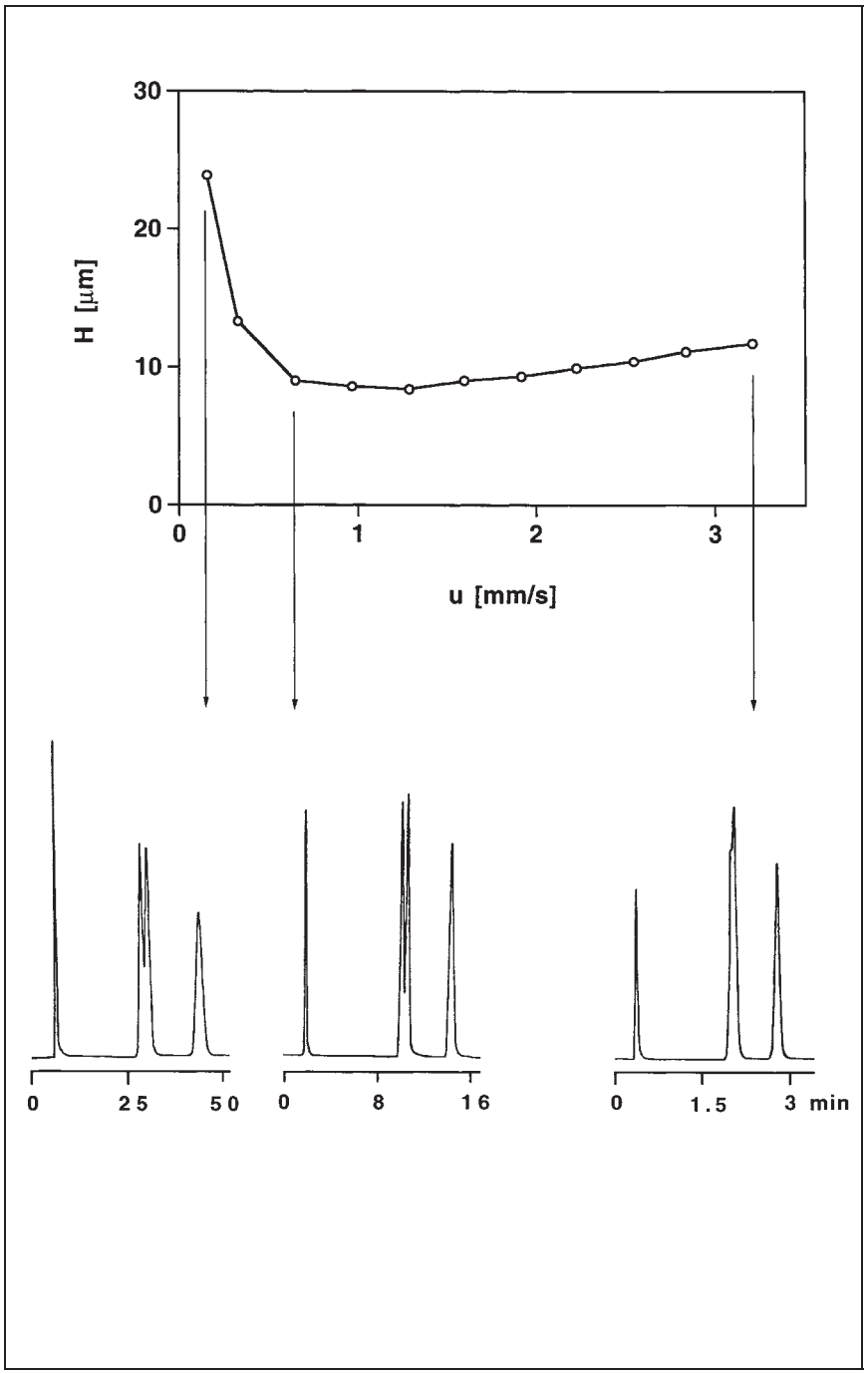
It would be best to work at u_{opt}. Practical separations are often performed at higher speed, which gives shorter analysis times and usually only a moderate loss of separation performance. This is, however, only true if mass transfer is fast, which is often not the case with special stationary phases and ion exchangers. Of course it is never advisable to work under conditions left of the van Deemter optimum. In this region the separation performance is very poor and the analysis time is long.

If the van Deemter curve is plotted with reduced parameters v and h the optimum is often at v = 3.

Chromatographic conditions:

Sample:	5 µL of a solution of thiourea, veratrole, acetophenone and nitrobenzene
Column:	4.6 mm × 7.5 cm
Stationary phase:	Zorbax SB C-18, 3.5 µm (reversed phase C_{18})
Mobile phase:	water/ethanol 3 : 7
Volume flow rate:	0.1 to 1.9 mL min^{-1} corresponding to u from 0.16 to 3.2 mm s^{-1}
Detector:	UV 268 nm
Optimum:	for nitrobenzene (curve) $u \approx 1.3$ mm s^{-1} (0.8 mL min^{-1}), $H \approx 8.5$ µm; for veratrole and acetophenone $u \approx 0.65$ mm s^{-1} (0.4 mL min^{-1})

Reference: V. R. Meyer


30
20
10
0
H [µm]
0
1
2
3
u [mm/s]
0
25
50
0
8
16
0
1.5
3 min

1.6 Peak Capacity and Number of Possible Peaks

For the separation of complex mixtures it is necessary to have space for as many peaks as possible with adequate resolution throughout the chromatogram. This number is the larger the higher the number of theoretical plates of the column and the longer one is willing to wait for the last peak. In other words, N and k determine the so-called peak capacity, n, of the column in use. Usually $R = 1.0$ is taken as the necessary resolution between two adjacent peaks (→ 1.3).

If the plate number were constant over the whole range of k values the peak capacity would be defined as:

$$n = 1 + \frac{\sqrt{N}}{4} \ln(1 + k_{\max})$$

At constant peak width, w, as could be the case with a very steep solvent gradient, the peak capacity would be much higher:

$$n = \frac{t_{\mathrm{R\,max}} - t_0}{w}$$

In practice neither plate numbers nor peak widths are constant. N increases with time because no chromatographic system has an ideal behavior; and even with gradient elution the peak widths increase gradually. Therefore the Number of Possible Peaks, NPP, is a more realistic value than the peak capacity, n:

$$\mathrm{NPP} = \frac{\Delta t_{\mathrm{R}}}{w_{\mathrm{n}} - w_1} \ln \frac{w_{\mathrm{n}}}{w_1}$$

with the retention time interval Δt_R covering peaks 1 to n (including their widths) and peak widths w.

The figure presents a first peak at 2 min, or 120 s, of width 6.2 s (N = 6000) and a last at 3.33 min, or 200 s, of width 8.4 s (N = 9000). Thus the retention time interval runs from (120-3.1) s to (200+4.2) s or a span of 87.3 s. With these data NPP = 12 as shown in the computer simulation with peaks of resolution 1.0.