

## 1 Evaluating Chromatograms

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Chromatography is, in principle, a dilution process. In HPLC analysis, on dissolving the substances to be analyzed in an eluent and then injecting 20  $\mu\text{l}$ , the peak volume exiting the column is greater than 20  $\mu\text{l}$ . This is a consequence of the chromatographic conditions. Depending on the column dimensions, there is a critical injection volume, which, if exceeded, leads to additional peak broadening. If one dissolves the substances to be analyzed in a weaker eluent, as is generally done when the gradient elution technique is used, then the injection volume that can be tolerated is increased significantly. The efficiency (Fig. 1.1) of the column is defined by the plate number  $N$  and the selectivity of the separation of two components is given by the separation factor  $\alpha$ .

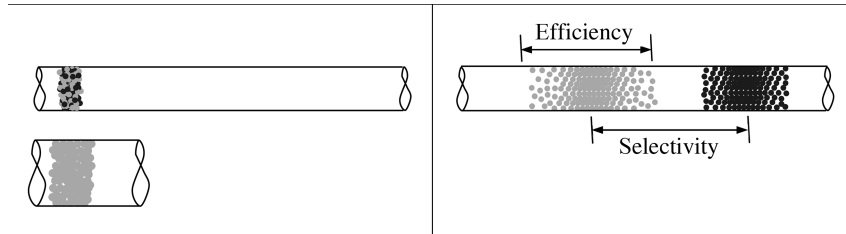
The **time taken for an eluent or carrier gas molecule to run through the column without retention is the mobile phase hold-up time**  $t_M$ . This often corresponds approximately to the time of the appearance of the first peak in the chromatogram. If one uses too strong an eluent, all components to be separated are eluted at the hold-up time because they are not held back on the column. Differences in the interactions of the substances with the stationary phase lead to different retention times,  $t_S$ , in the stationary phase. To achieve a separation,  $t_S$  must be greater than  $t_M$ .

$$t_R = t_M + t_S \quad (1.1)$$

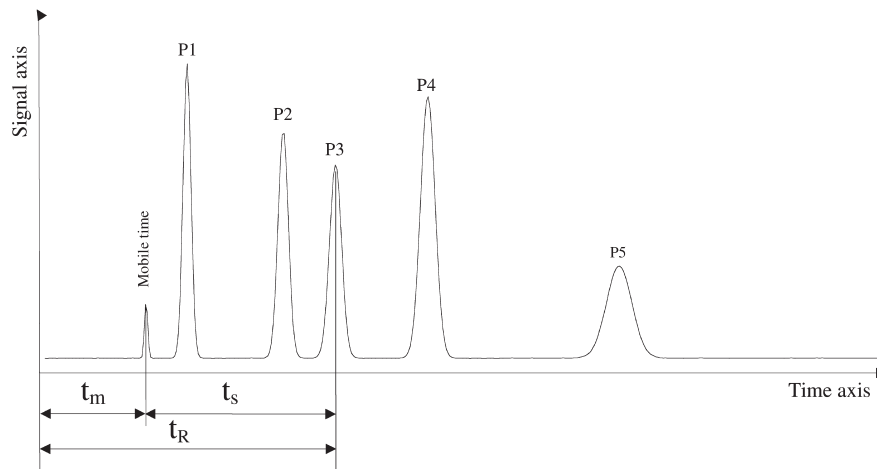
$$k = \frac{t_S}{t_M} \quad (1.2)$$

$$u = \frac{L}{t_M} \quad (1.3)$$

The sum of  $t_M$  and  $t_S$  yields the measured retention time  $t_R$  (Fig. 1.2) and the quotient of  $t_S$  and  $t_M$  is the retention factor  $k$ , which is not dependent on the column dimensions or the flow, as is  $t_R$ . With increasing pressure the flow and



**Figure 1.1** Efficiency is dependent on the peak width, selectivity on the retention factor quotient.



**Figure 1.2** Retention times are measured at the apex of each peak. Unretained components elute at the mobile phase hold-up time.

the linear velocity increase in parallel. The linear velocity  $u$  is calculated by dividing the column length,  $L$ , by  $t_M$ .

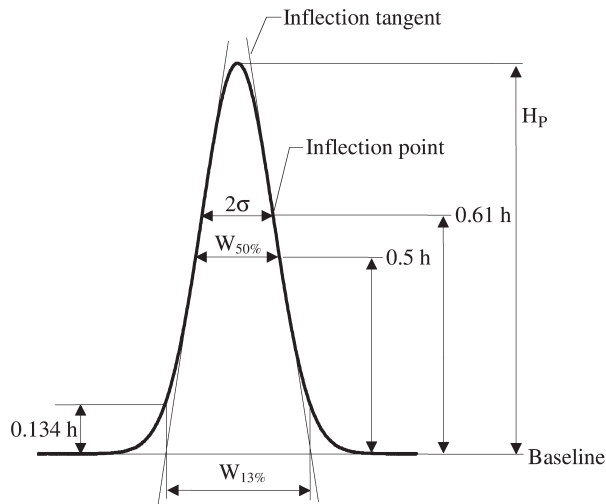
### 1.1 Efficiency

The ideal peak shape is described by a symmetric Gaussian peak (Fig. 1.3), characterized by the retention time  $t_R$  and the standard deviation  $\sigma$  of the retention time, which is a measure of the peak width.

$$N = \frac{t_R^2}{\sigma^2} \quad (1.4)$$

$$w_{50\%} = 2.35 \sigma \quad (1.5)$$

$$w_{13\%} = 4 \sigma \quad (1.6)$$



**Figure 1.3** Characteristic parameters of a Gaussian peak.

Besides the well known graphic methods,  $N$  can be calculated using the area  $A$  and height  $H$ :

$$N = 2 \pi \left( \frac{t_R H_P}{A} \right)^2 \quad (1.7)$$

Combining Eqs. (1.4) and (1.7) it can be seen that  $\sigma$  can be determined from the retention time, area and height.

$$\frac{t_R^2}{\sigma^2} = 2 \pi \frac{t_R^2 H_P^2}{A^2} \quad (1.8)$$

$$\sigma = \frac{A}{H_P \sqrt{2 \pi}} \quad (1.9)$$

These equations only apply to isocratic separations. For low  $k$  values, one usually finds smaller plate numbers than for higher  $k$  values. The extra column volume  $V_{ec}$  leads to an additional constant peak broadening. The smaller earlier peaks are influenced more strongly by this than the later peaks.

## 1.2 EMG Model

In practice, peaks often have a tailing, which can be described as a Gaussian peak with an overlaid exponential function (exponential modified Gauss function

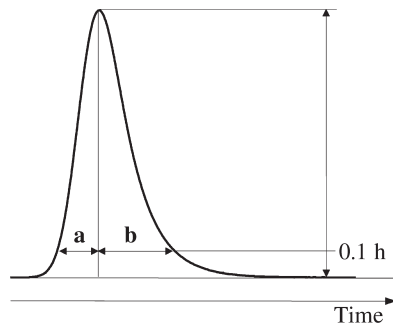


Figure 1.4 The asymmetry factor is measured at 10% of the height.

(EMG) [1]). The increase in the Gaussian peak is influenced less by the exponential tail with the time constant  $\tau$  than by the decreasing portion of the peak. The retention time is shifted to a slightly higher value, as is the peak width.

$$N = \frac{t_R^2}{\sigma^2 + \tau^2} \quad (1.10)$$

The values of  $\sigma$  and  $\tau$  are not determined by the integration method used. The extent of the tailing, which is measured at 10% of the peak height, is represented by the asymmetry factor  $A_f$  (Fig. 1.4).

$$A_f = \frac{a}{b} \quad (1.11)$$

According to the EMG model, the plate number is calculated as:

$$N = 41.7 \frac{\left[ \frac{t_R}{a+b} \right]^2}{\frac{b}{a} + 1.25} \quad (1.12)$$

In the range  $\tau/\sigma = 0.5-3$  Tamisier-Karolak [2] found an empirical equation:

$$\frac{\tau}{\sigma} = -1.03 \left( \frac{b}{a} \right) - 0.97 \quad (1.13)$$

This equation can only be used if the asymmetry factor can be estimated. Therefore the valley between the peaks must not be greater than 9% of the height of the smaller peak. In practice, the asymmetry factor at the peak's half height is of more use. If the valley between the peaks is greater than 49%, integration with the perpendicular drop method is very prone to errors, because the second peaks sits on the first peak.

The plate number is dependent on the column length and the particle size. If one divides the column length by the plate number, the plate height  $H$  ( $\mu\text{m}$ ) is obtained. Dividing  $H$  by the particle size  $d_p$ , one obtains the reduced plate height  $h$ , i.e. the number of particles due to one plate.

$$H = \frac{L}{N} \quad (1.14)$$

$$h = \frac{H}{d_p} \quad (1.15)$$

In theory, the minimum possible value for  $h$  is 2, but in practice, a value of 3 is usually acceptable in real chromatograms.

The three effects: flow inhomogeneity  $A$ , diffusion broadening  $B$  in competition with the flow and deterioration of the component exchange  $C$  with increasing flow are overlaid, to produce a Van Deemter  $Hu$ -curve. The essential statement is that there is a linear velocity  $u$  at which the plate height  $H$  has a minimum. A column has the highest separating performance at this flow, which is characterized by the linear velocity  $u_{\text{opt}}$ . In HPLC one usually works above the optimal flow, accepting a loss in the optimal separating performance because otherwise the analysis times would become too long. The corresponding broadening of the peak is particularly dependent on the component exchange term  $C$ , which causes the increasing slope of the  $Hu$  curve.

### 1.3 Chromatogram

A chromatogram is a graphical representation of all peaks eluting from the column superimposed on the baseline. The areas and heights of the peaks usually increase linearly in accordance with the amount of injected component. The integration systems enable an automated estimation of the area, height and other characteristic parameters.

The separation is usually carried out on a C18 column in HPLC and on a capillary column in GC. The separating ability of a column is characterized by the plate number, which determines the peak width relative to the retention time. A typical value for HPLC is 10 000 and for GC 90 000 plates.

$$\frac{s_p}{t_R} = \frac{1}{\sqrt{N}} \quad (1.16)$$

If the standard deviation of the peak is 1% of the retention time  $t_R$ , the plate number is 10 000.

## 1.4

## Selectivity

The aim of chromatography is a separation, which is characterized by the different retention times of two peaks succeeding one another. A very efficient column with a high plate number is no guarantee of an efficient separation. The selectivity is characterized by the separation factor  $\alpha$ , which is the quotient of the two retention factors.

$$\alpha = \frac{k_2}{k_1} \quad (1.17)$$

An  $\alpha$  of 1.1 implies that the retention time  $t_s$  of the second peak is 10% longer than the retention time of the first peak. The resolution  $R$ , a measure of the quality of the separation between two peaks, depends not only on the distance between the two peaks but also on the peak width, which can be found graphically as the peak width at half height  $w_{50\%}$ .

$$R = 1.18 \frac{t_{R2} - t_{R1}}{w_{250\%} + w_{150\%}} \quad (1.18)$$

An  $R$  of 1.5 is known as baseline separation, although only  $R = 2$  is really separation to the baseline.

If the retention times of two successive peaks lie sufficiently far away from each other then we have a separation down to the baseline. If not, the peaks merge together and the valley between them decreases until only a broadened peak can be seen. One does not have any chance of recognizing whether several components hide under the visible peak at (almost) the same retention time. This can be resolved only by variation of the separating conditions or with one or more specific detectors.

## References

- 1 J. P. Foley, J. G. Dorsey, Equations for Calculation of Chromatographic Figures of Merit for Ideal and Skewed Peaks, *Anal. Chem.* 55, 1983, 730–737.
- 2 S. L. Tamisier-Karolak, M. Tod, P. Bonnardel, M. Czok, P. Cardot, Daily validation procedure of chromatographic assay using gaussoexponential modelling, *J. Pharm. Biomed. Anal.* 13, 1995, 959–970.