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A Guide to Validation in HPLC

Based on the work of G. M. Hearn PERKIN ELMER

1. Introduction

Sometimes you may wonder who was the first to make an experimental procedure for a specific analysis. The route from an idea to an actual standard operating procedure (SOP) is not easy. In the early days it was a long period of trial and error to establish a document where common users could benefit from. Nowadays the use of computer programmes like Drylab or HIPAC makes it easier to optimize the intended procedure to a practical analysis for other laboratories and schools. This document will try to explain some of the basic jargon and glossary concerning HPLC. At the end you may understand the long route to a method that has been validated as have many of the experiments and analytical procedures you will use within StandardBase.

2 METHOD VALIDATION

"Validation of an analytical method is the process **by** which it is established **by** laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical application - **"(6)**

Validation is required for any new or amended method to ensure that it is capable of giving reproducible and reliable results, when used by different operators employing the same equipment in the same or different laboratories. The type of validation programme required depends entirely on the particular method and its proposed applications.

Typical analytical parameters used in assay validation include:

1. Precision
2. Accuracy
3. Linearity
4. Range
5. Ruggedness
6. Limit of detection
7. Limit of quantitation
8. Selectivity
9. Specificity

2.1 Precision

> "The precision of an analytical method is the degree of agreement among individual test results obtained when the method is applied to multiple sampling of a homogenous sample **-"(6)**

Precision is a measure of the reproducibility of the *whole* analytical method (including sampling, sample preparation and analysis) under normal operating circumstances. Precision is determined by using the method to assay a sample for a sufficient number of times to obtain statistically valid results (ie between 6 - 1 0). The precision is then expressed as the relative standard deviation

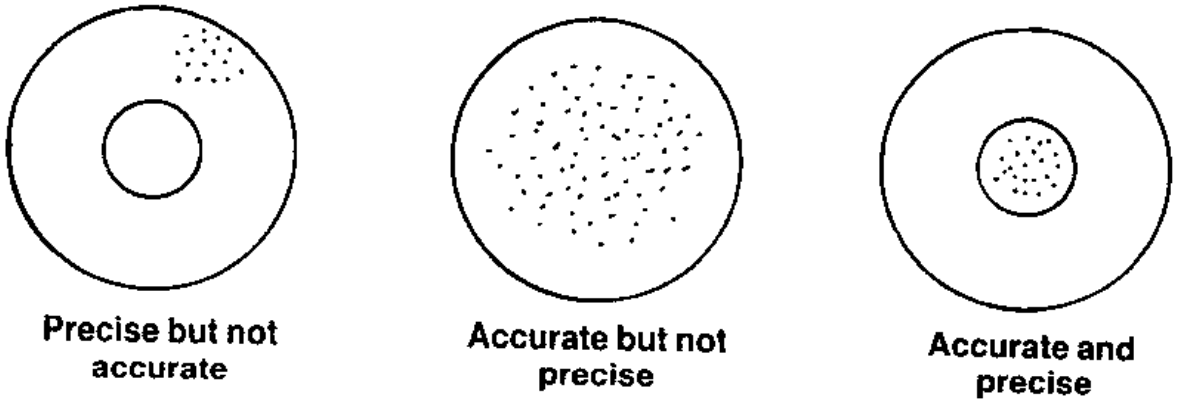
$$\%RSD = \frac{\text{std dev} \times 100\%}{\text{mean}}$$

2.2 Accuracy

> "Accuracy is a measure of the closeness of test results obtained by a method to the true value. " (6)

Accuracy indicates the deviation between the mean value found and the true value. lt is determined by applying the method to samples to which known amounts of analyte have been added. These should be analysed against standard and blank solutions to ensure that no interference exists. The accuracy is then calculated from the test results as a percentage of the analyte recovered by the assay.

Accuracy and precision are not the same, as the diagram below indicates. A method can have good precision and yet not be accurate.



Errors in measurement can be divided into two general categories: systematic errors and

random errors.

Systematic errors result from sources that can be traced to the methodology, the instrument or the operator, and affect both the accuracy and the precision of the measurement.

Random errors only affect the precision, and are difficult to eliminate, because they are the result of random fluctuations in the measured signal, due to noise and other factors.

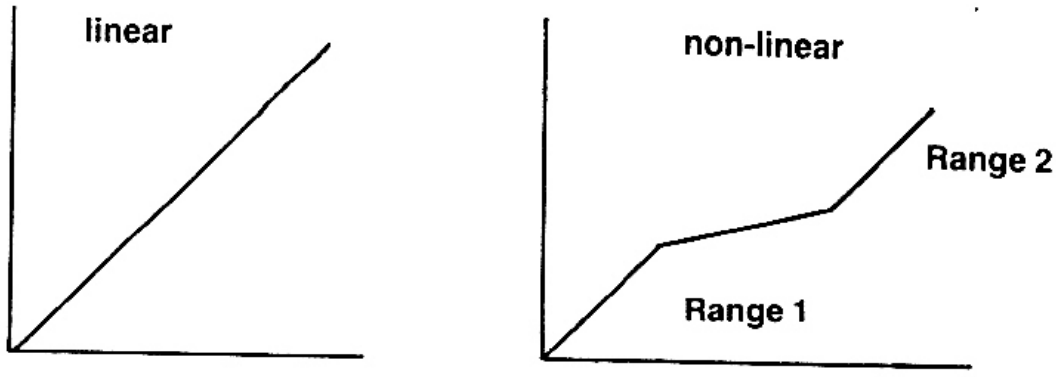
Whilst systematic errors are proportional to the sum of individual contributions, random errors are proportional to the root of the sum of the squares of the individual contributions. Thus, the imprecision of the entire procedure is often dominated by the random errors of the most imprecise step.

2.3 Linearity

This is the method's ability to obtain results which are either directly, or after mathematical transformation proportional to the concentration of the analyte within a given range.
Linearity is determined by calculating the regression line using a mathematical treatment of the results (ie least mean squares) vs analyte concentration.

2.4 Range

The range of the method is the interval between the upper and lower levels of an analyte that have been determined with acceptable precision, accuracy and linearity. It is determined on either a linear or nonlinear response curve (ie where more than one range is involved, as shown below) and is normally expressed in the same units as the test results.



2.5 Ruggedness

Ruggedness is the degree of reproducibility of results obtained by the analysis of the same sample under a variety of normal test conditions ie different analysts, laboratories, instruments, reagents, assay temperatures, small variations in mobile phase, different days etc. (ie from laboratory to laboratory, from analyst to analyst.)

2.6 Limit of Detection

This is the lowest concentration in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. The limit of detection is important for impurity tests and the assays of dosages containing low drug levels and placebos. The limit of detection is generally quoted as the concentration yielding a signal-to-noise ratio of 2:1 and is confirmed by analyzing a number of samples near this value (6) using the following equation. The signal-to-noise ratio (5) is determined by:

$$s = H/h$$

Where H = height of the peak corresponding to the component
h = absolute value of the largest noise fluctuation from the baseline of the chromatogram of a blank solution.

Since the limit of detection is dependant on the signal-to-noise ratio, it can be improved by enhancing the analyte signal and reducing the detector noise.

The signal (ie peak height) can be increased by selecting the optimum monitoring wavelength, increasing the injection volume or mass (below signal or column saturation), increasing the peak sharpness with high efficiency columns and by optimizing the mobile phase. For absorbance detectors, longer path lengths in the flow cell enhances sensitivity though often to the detriment of post column dispersion.

Noise can be reduced by using high sensitivity detectors with low noise and drift characteristics, slower detector response time, mobile phases with low absorbance and pumps with low pulsation.

2.7 Limit of Quantitation

This is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy.

lt is quoted as the concentration yielding a signal-to-noise ratio of 1 0: 1 and is confirmed by analyzing a number of samples near this value (5).

2.8 Selectivity and Specificity

Selectivity is the ability to measure accurately and specifically the analyte in the presence of components that may be expected to be present in the sample matrix.

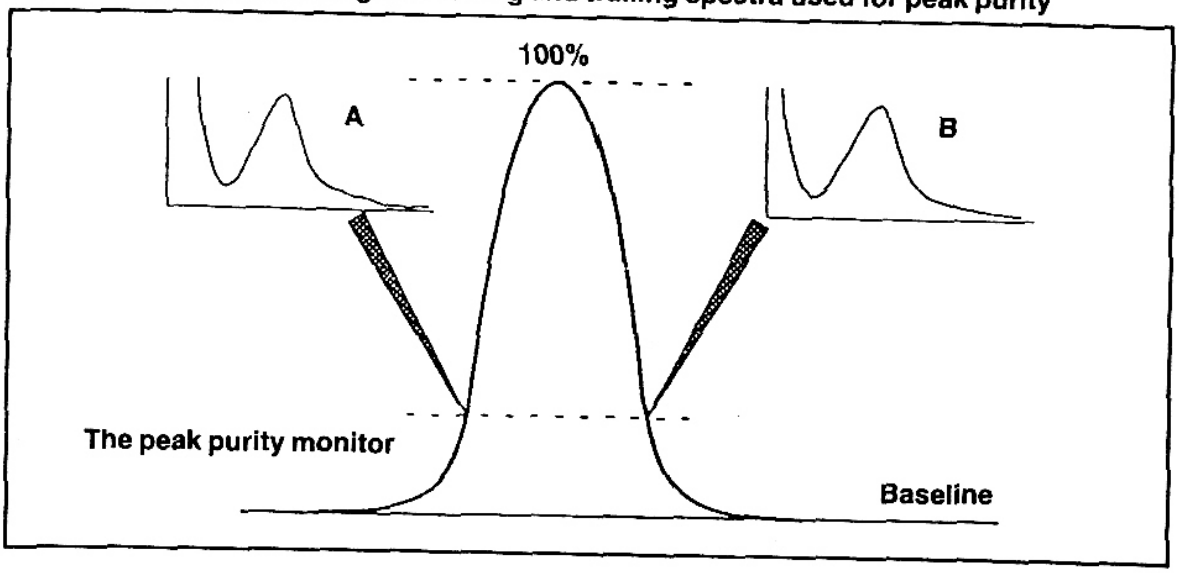
Specificity for an assay ensures that the signal measured comes from the substance of interest, and that there is no interference from excipient and/or degradation products and/or impurities.

Determination of this can be carried out by assessing the peak identity and purity.

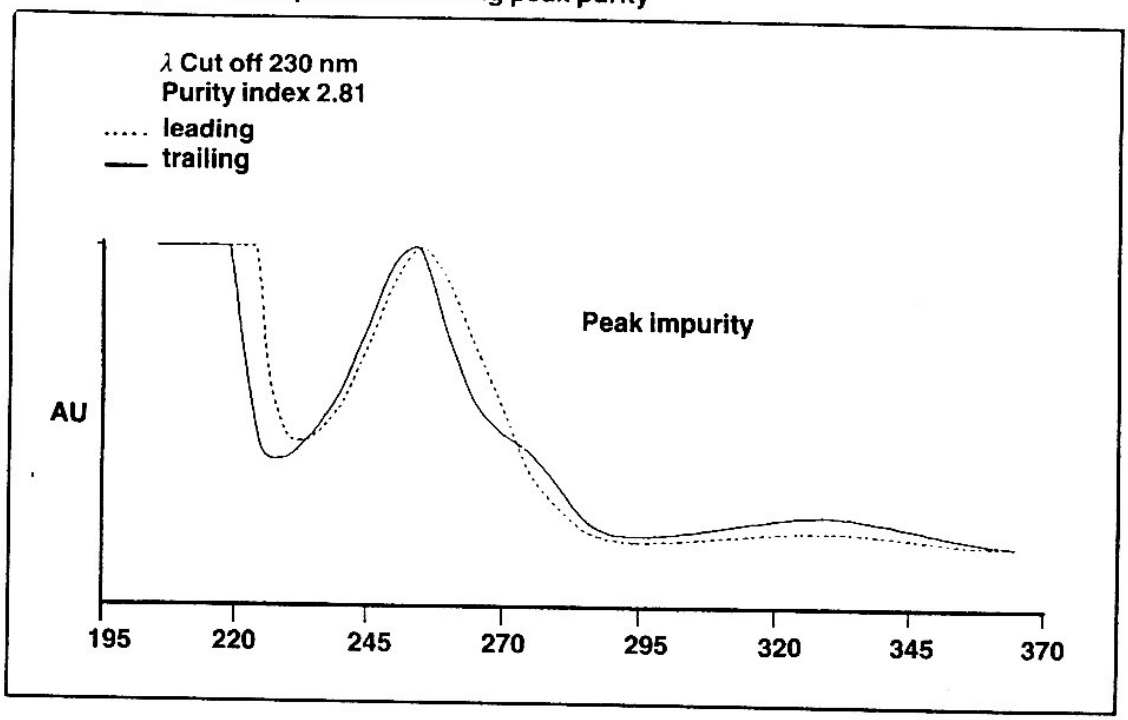
Diode array detectors can facilitate the development and validation of HPLC assays. Spectra] data obtained from diode array detectors, effectively supplement the retention time data for peak identification, also spectral manipulation often provides information about the peak purity. The table below lists several of the techniques available for assessing peak identity and purity.

The purity index is a measure of the peak's relative purity, measured using a full comparison of spectral data for the leading and training edge of the peak Figure 4. A value of 1.5 is commonly accepted to indicate a pure peak but >1.5 would indicate the presence of an impurity, (9) as shown in Figure 5.

Figure 4: Diagram indicating the leading and trailing spectra used for peak purity

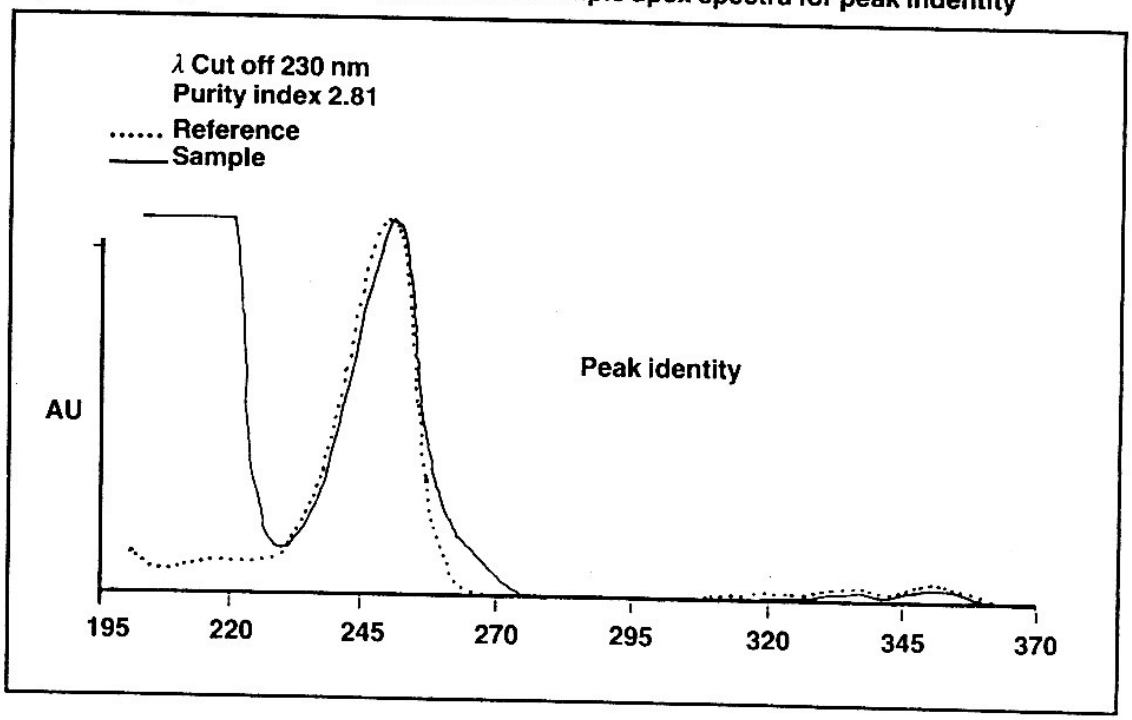


gure 5: Spectral comparison showing peak purity



The peak identification (9) is a comparison of the apex spectrum against that of a reference (stored under the same conditions).

Figure 6: Comparison of reference versus sample apex spectra for peak indentity



3. SYSTEM SUITABILITY TESTS (SST)

Once a method or system has been validated the task becomes one of routinely checking the suitability of the system to perform within the validated limits.
The simplest form of an HPLC system suitability test involves a comparison of the chromatogram trace with a standard trace (as shown below). This allows a comparison of the peak shape, peak width, baseline resolution.
Alternatively these parameters can be calculated experimentally to provide a quantitative system suitability test report:

Number of theoretical plates (efficiency)

Capacity factor,

Separation (relative retention)

Resolution,

Tailing factor

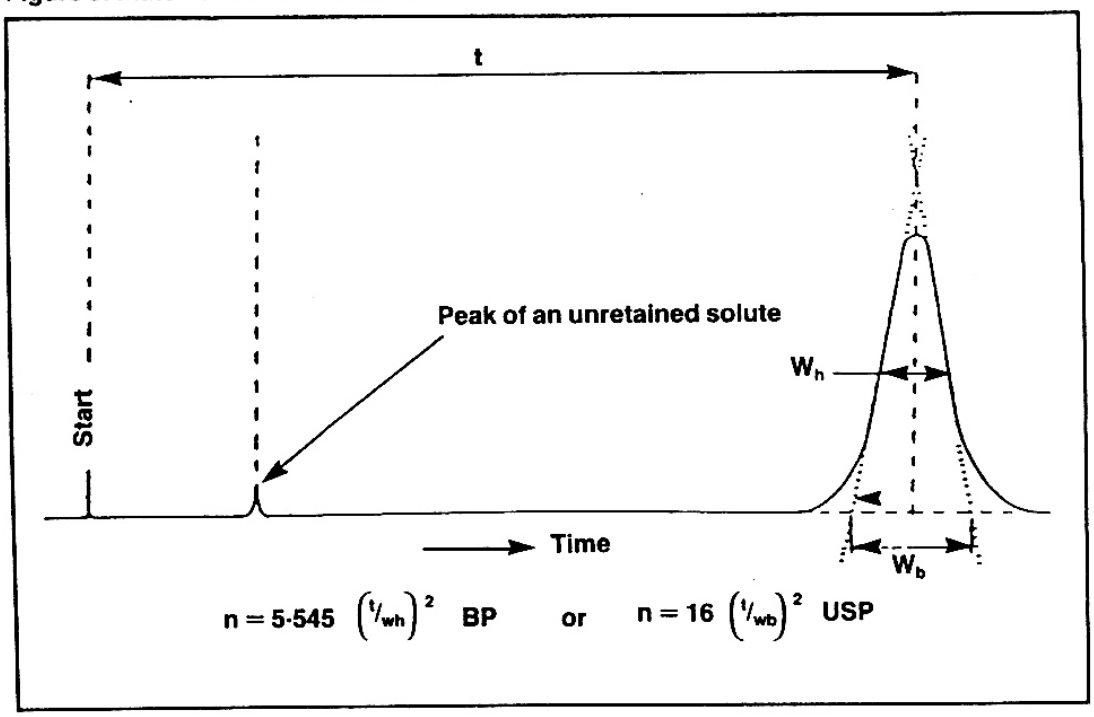
Relative Standard Deviation (Precision)

These are measured on a peak or peaks of known retention time and peak width.

3.1 Plate number or number of theoretical plates (n)

This a measure of the sharpness of the peaks and therefore the efficiency of the column. This can be calculated in various ways, for example the USP uses the peak width at the base and the BP at half the height.

Figure 9: Plate number calculation



where
Wh = peak width at 1/2 peak height
Wb = peak width at base
t = retention time of peak

Therefore the higher the plate number the more efficient the column.

The plate number depends on column length - ie the longer the column the larger the plate number. Therefore the column's efficiency can also be quoted as:

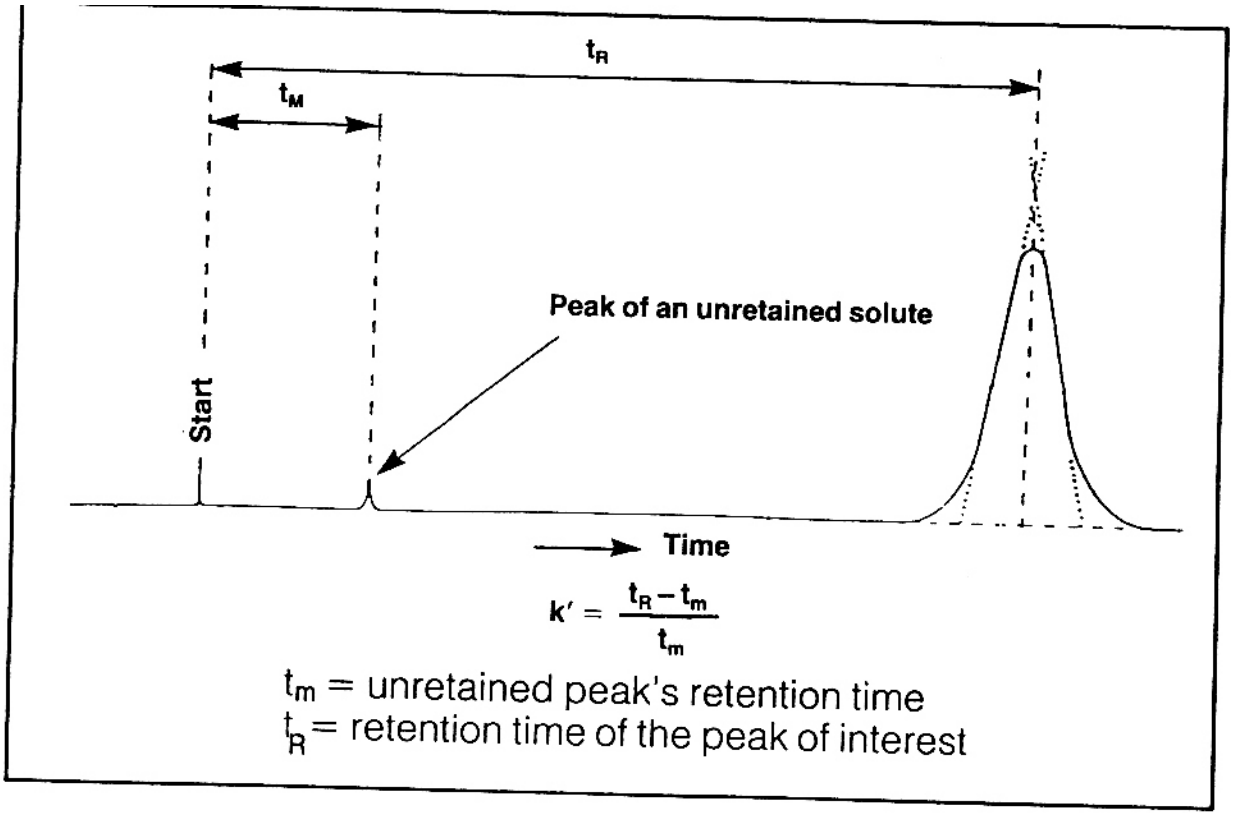
Either- as the plate height (h), **or** the height equivalent to one theoretical plate (HETP).

$$h = \frac{L}{n}$$ where L = length of column n

Or- as plates/meter.

3.2 Capacity factor (capacity ratio) k

This value gives an indication of how long each component is retained on the column (ie how many times longer the component is retarded by the stationary phase than it spends in the mobile phase).



k' is used in preference to retention time because it is less sensitive to fluctuations in chromatographic conditions (ie flow rate) and therefore ensures greater reproducibility from run to run.

In practice the k value for the first peak of interest should be >l to assure that it is separated from the solvent.

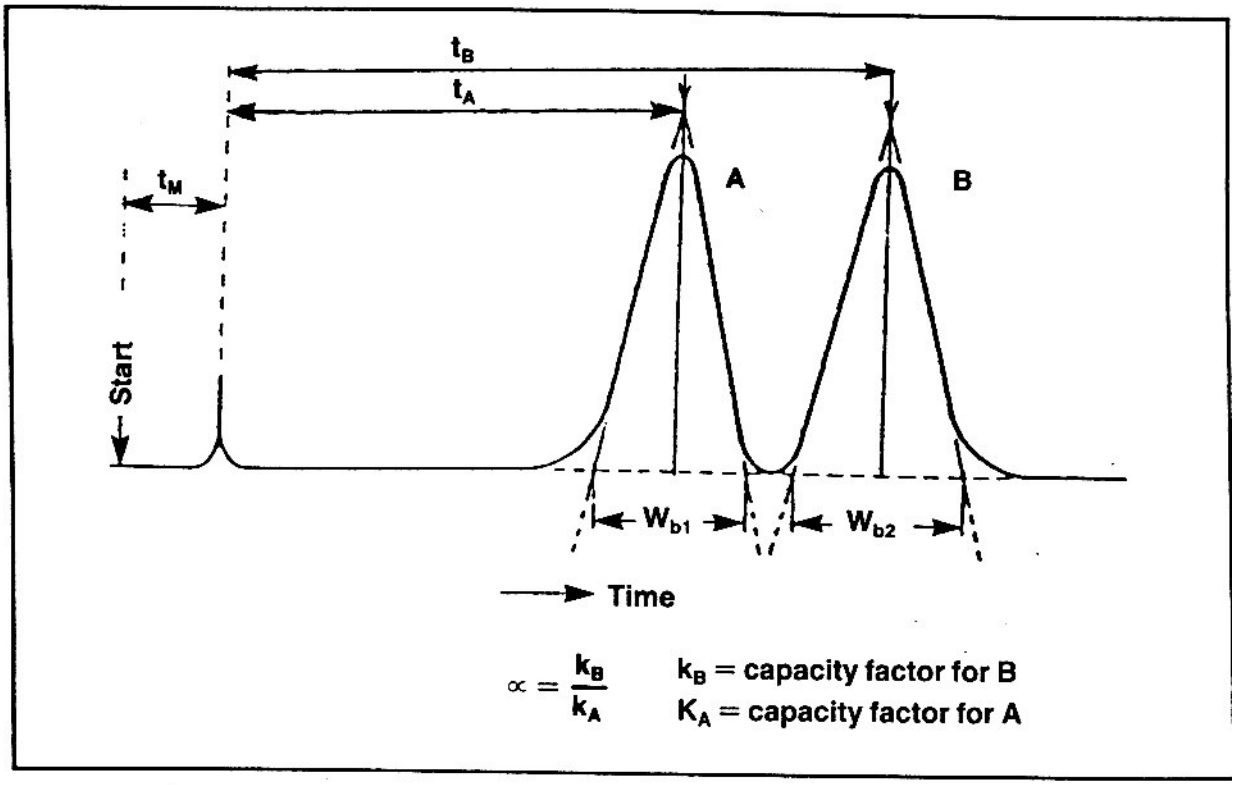
3.3 Separation Factor (relative retention)

This describes the relative position of two adjacent peaks. Ideally, it is calculated using the capacity factor because the peaks' separation depends on the components' interaction with the stationary phase.

Therefore considering peaks A and B

Figure 1 1: Separation factor calculation

Figure 11: Separation factor calculation



k for the later peak is always placed in the numerator to assure a value >l.

If the capacity factor is used then the separation factor should be consistent for a given

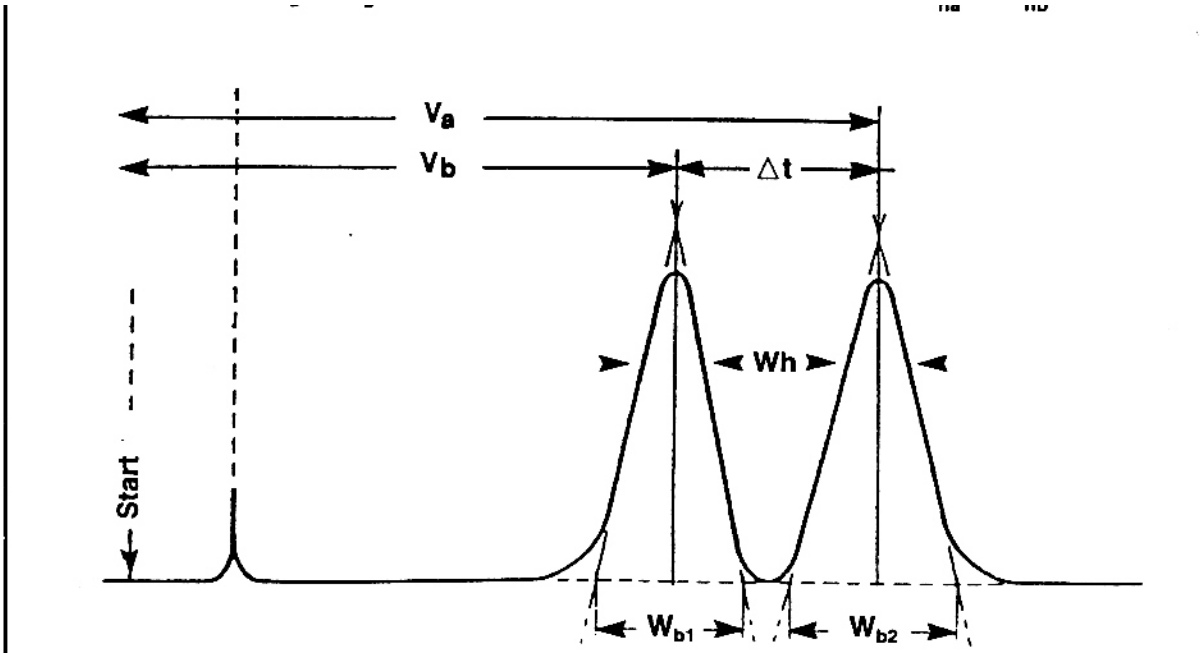
column, mobile phase, composition and specified temperature, regardless of the instrument used.

NB. The separation factor gives no indication of the efficiency of the column.

3.4 Peak Resolution R

This is not only a measure of the separation between two peaks, but also the efficiency of the column. It is expressed as the ratio of the distance between the two peak maxima. (At) to the mean value of the peak width at base (Wb).

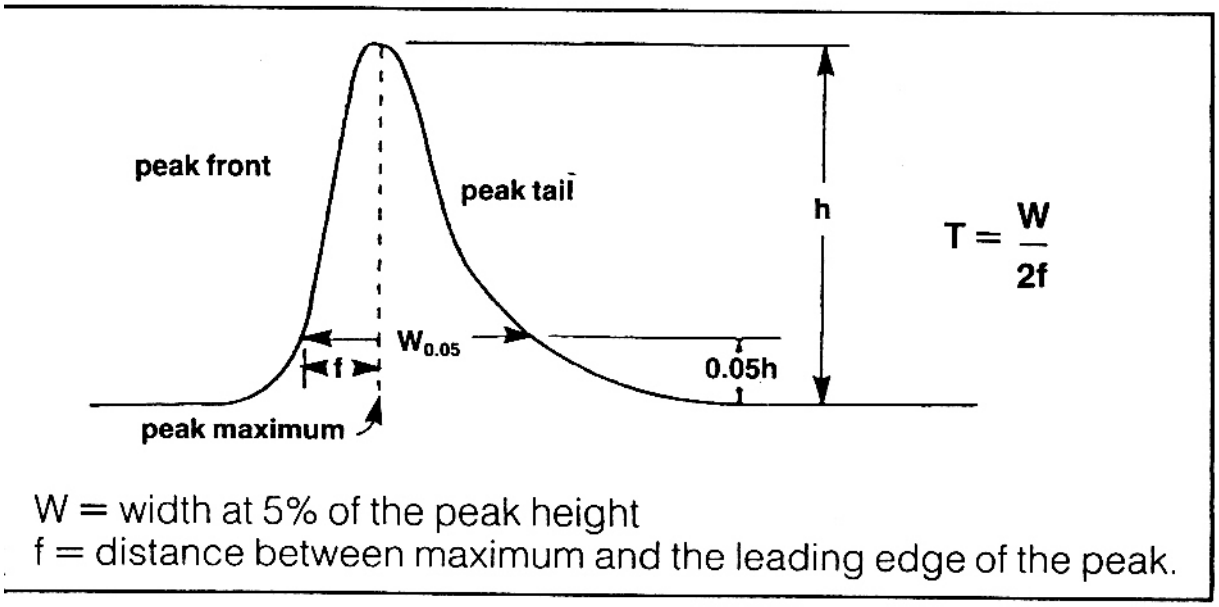
Figure 12: Peak resolution calculation



3.5 Tailing factor T

This is a measure for the asymmetry of the peak.

Figure 13: Tailing factor calculation



3.6 Relative Standard Deviation or precision

For an HPLC system this would involve the reproducibility of a number of replicate injections (ie 6) of an analytical solution.

The USP requires that unless otherwise specified by a method:

- if a relative standard deviation of <2% is required then five replicate injections should be used

- if a relative standard deviation of >2% is required then six replicate injections should be used

Tabie 5 below lists factors w,hich could affect the precision of an HPLC system.

Tabie 5: Factors affecting LC system **precision**

Precision	**Controlling Factors**
Retention time	Pump f low and composition precision Column temperature Mobile phase composition
Peak area	Autosampler: inj mode, inj volume Pump: flow, pulsation Detector: noise and drift, response Data system: sampling rate, integration parameters

In most cases the system's Relative Standard Deviation is required; deciding which of the other tests are required is not straightforward. To assist with the decision it has been suggested that those parameters which have an affect on the system precision should be used. For instance the resolution of two peaks with similar retention times should be quoted, because, if it is below a critical value, the precision will be affected.

In addition "diode array" detectors allow for the determination of the relative purity factor typically called: Peak Purity.

The retention time precision is important, because not only is retention time the primary method for peak identification, but also variations can indicate problems within the LC system (ie with the piston seals, check valves etc). Use of a column oven can overcome laboratory temperature variations, which is the most common cause of retention time drift.

The most dominant factor controlling the repeatability of peak area is the autosampler's precision, though the affect of noise and integration parameters will become more significant with small peaks.

CONCLUSION

Reproducible quality HPLC results can only be obtained if attention has been paid to the method development, validation and the system's suitability to carry out the analysis.

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10 http://www.standardbase.com for more experiments.