

Column efficiency testing

Packed bed chromatography is a versatile separation technique frequently used in the purification of biopharmaceuticals. The preparation and qualification of packed columns are important steps to ensure robustness and safety for both the purification process and the final product. Column efficiency testing plays a central role in the qualification and monitoring of packed bed performance. Even though it cannot be used as a single parameter to predict purity and recovery, it is a quick way to test the column and equipment performance before starting the purification process. This test can also be used inbetween runs to check for changes of the bed integrity. This note provides a brief overview of the theory behind and experimental test practices used in column efficiency testing. Test conditions are recommended and critical parameters that influence the measured efficiency are discussed in order to facilitate the development of robust test protocols.

Theoretical background

Efficiency testing is the analysis of the residence time distribution for a tracer substance passing through the column. Typical test signals applied to the column are pulse or step signals. In order to characterize the chromatography column without interference, tracer substance and eluent conditions are selected such that chemical interactions with the medium and disturbances of the fluid flow are avoided.

Pulse Test

The most common type of test signal applied is a pulse function. A small volume of a tracer substance is added to the liquid flow close to the column inlet and the broadening of this pulse is analyzed when measured as a chromatographic peak at the column outlet. A typical pulse response and its evaluation are summarized in Figure 1.

Column efficiency is typically defined in terms of two parameters:

- Peak broadening over the column is described by an equivalent number of theoretical plates (equilibrium stages)
- Peak symmetry is described by a peak asymmetry factor A_s

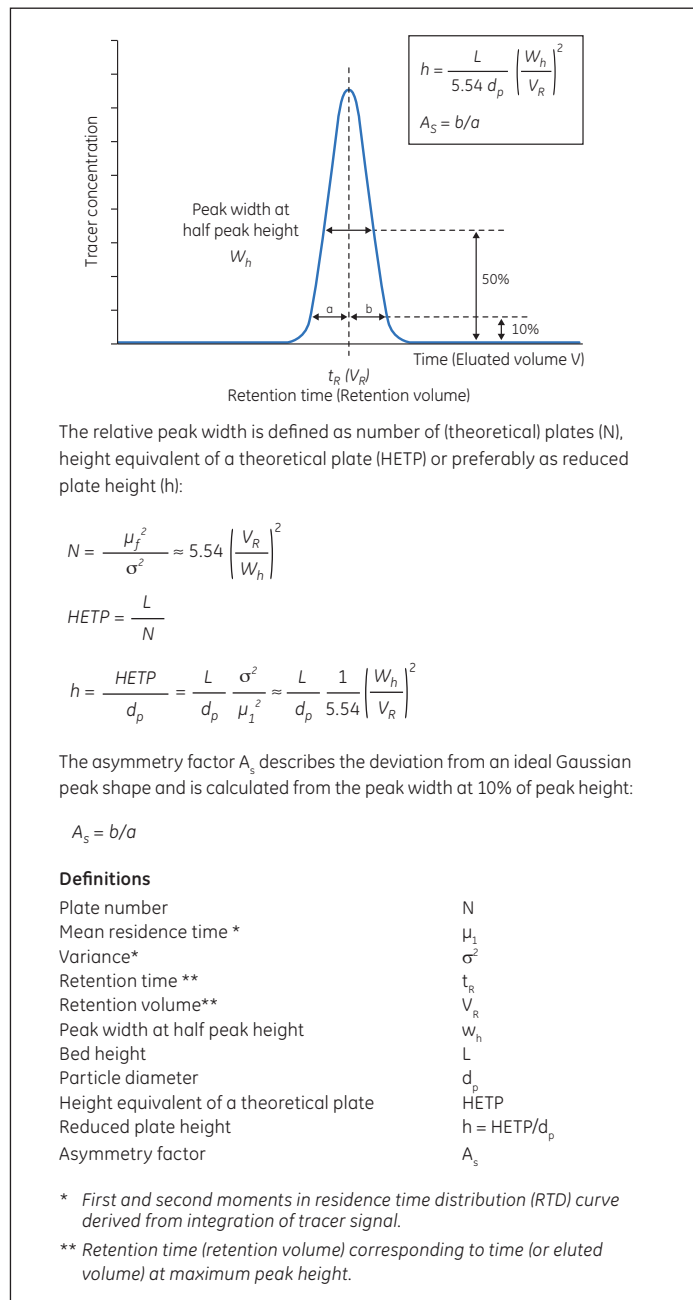


Fig 1. Schematic overview of the pulse test with calculations and definitions.



Simplified peak evaluation by measurement of peak width and asymmetry factor

Peak broadening is typically described as plate number N or as height equivalent to a theoretical plate (HETP). This concept is equivalent to a tanks-in-series model reflecting the number of equilibrium stages represented by the column.

A widely used method for evaluating a pulse test (to determine the plate number) involves the measurement of peak width at half of the maximum peak height. This approach is an alternative to numerical curve integration when applying the method of moments, where the first moment is the average and the second moment is the variance of the retention volume/time. As outlined in Figure 1, the pulse response is plotted against time or eluted volume and the peak width at half peak height is measured and related to the elution time or, preferably, the eluted volume at maximum peak height. The retention time or retention volume measured at the maximum peak height corresponds to the average residence time or volume found at a symmetric (Gaussian) peak shape.

A dimensionless and thus convenient parameter for efficiency characterization is the reduced plate height h . This parameter facilitates the comparison of column efficiency irrespective of column length and particle diameter of the medium.

The measurement of the ascending and descending portions of the peak at 10% of the peak height is a standard chosen to allow for simple evaluation. Deviations from an ideal peak symmetry ($A_s = 1$) may be caused by irregularities in the packed bed itself, but also by unfavorable fluid flow within tubing and components external to the packed bed. In addition, peak asymmetry may arise from air trapped within the column or from clogged frits or screens in the fluid distribution system, resulting in flow disturbances.

Optimal column efficiency

Optimal column efficiency typically corresponds to an experimentally determined reduced plate height of $h \leq 3$ for the porous media employed in bioprocess chromatography. This efficiency is achieved when testing a well-packed bed with an optimized set-up of column and system under optimal test conditions. In practice, acceptance criteria often need to be adjusted (e.g., limitations in available hardware). A high-throughput production system operating at high velocity during processing is probably not appropriate for running an efficiency test at optimal conditions.

Deviations from optimal column efficiency may be acceptable depending on the specific application. In order to enable qualification of all chromatography applications including high-resolution gel filtration and challenge polishing chromatography, recommendations made by GE Healthcare are usually aimed towards this high efficiency specification.

Asymmetry

In regard to the peak asymmetry, an asymmetry factor close to $A_s = 1$ is ideal. A typical acceptable range could be $0.8 < A_s < 1.8$ when working towards a reduced plate height of $h \leq 3$. It should be kept in mind that lower efficiency in terms of

peak width generally provides a more symmetric appearance of the peak. However, difficulties in observing and correcting imperfections in column packing or unfavorable set-up of column and system may arise.

Selecting appropriate test conditions

In theory, the best column efficiency achievable in terms of reduced plate height is typically $h = 1.5$ to 2 when using porous chromatography media used in bioprocess applications. This theoretical efficiency is derived from a Van Deemter analysis and reflects the convective and diffusive dispersion effects within the packed bed under optimized test conditions (i.e., liquid velocity). Additional sources of peak broadening are caused by packed bed heterogeneity, imperfections in column fluid distribution, system effects, or sample volume. In order to set appropriate specifications for chromatography column testing (and also for troubleshooting purposes) it is important to control these individual sources of band broadening that define the baseline for the theoretical efficiency.

Tracer substance

Tracer substances should be selected such that their size (molecular weight) allows for full penetration of the porous structure of the chromatography medium. Surface interactions of the tracer substance with the chromatography medium must be avoided to ensure the tracer is chemically inert and stable. In addition, viscosity and density differences between eluent and sample should be kept to a minimum to avoid systematic errors due to unstable flow patterns (so-called viscous fingering effects).

Commonly used sample/eluent systems that fulfill these requirements are acetone in water, and salt systems, which are monitored by measurement of absorbance and conductivity, respectively. Depending on the chemistry of the chromatography medium, the following sample and eluents are recommended:

For all media, except for hydrophobic synthetic polymers, RPC and HIC media:

Eluent: water
Sample: 1% - 2% acetone in water

For RPC and HIC:

Eluent: 20% ethanol
Sample: 1% - 2% acetone in at least 20% ethanol

For all media:

Eluent: 0.4 M NaCl in water
Sample: 0.8 M NaCl in water

For the use of NaCl as a tracer, a minimum of 0.4 M NaCl (35 - 40 mS/cm) in the eluent has to be used to suppress charge interaction effects between the tracer and the chromatography medium (otherwise, misleading test results may be observed). An example of the disturbance of peak symmetry when using a tracer substance that interacts with the medium is seen in Figure 2.

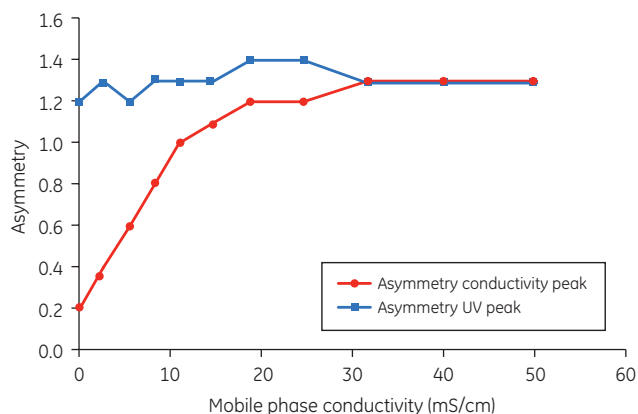


Fig 2. Effect of the mobile phase conductivity on the asymmetry of the conductivity peak in pulse evaluation of a packed bed of Sepharose™ XL. The bed was tested with a sample containing 1% acetone and 1 M NaCl (sample volume 1% V_c). The sample concentrations were kept constant while the conductivity in the eluent was changed. The UV-pulse asymmetry was between 1.2 and 1.4 in all runs. The conductivity pulse was strongly affected by the mobile phase conductivity. If the conductivity of the eluent is too low, a leading peak will be observed.

Liquid velocity

Peak broadening and therefore chromatographic efficiency is strongly dependent on the liquid velocity applied during the test. The relationship between peak broadening and liquid velocity is described theoretically by the Van Deemter equation (1):

$$HETP = A + \frac{B}{u} + C \times u$$

where A is related to eddy dispersion

B is related to molecular diffusion

C is related to mass transfer resistance

u is liquid velocity (cm/h)

An experimental analysis illustrating the impact of liquid velocity on column efficiency is shown in Figure 3. A minimum for the reduced plate height and highest column efficiency can be found for a liquid velocity around 20 cm/h. At lower velocities, the efficiency is reduced by means of molecular diffusion corresponding to term B in the Van Deemter equation. At high test velocities, and thus shorter residence time over the column, peak broadening is increasing as result of limiting intraparticle diffusion as represented by term C in the Van Deemter equation. Also note that peak asymmetry in Figure 3 decreases with higher liquid velocity. This is primarily attributable to a broadening peak diminishing the impact of asymmetry introduced by the test system setup (as described above).

The acceptance criterion of $h \leq 3$ assumes the use of an appropriate liquid velocity to achieve the optimal theoretical efficiency. Particle size (the characteristic length for diffusion) is a major parameter determining optimal liquid velocity (see Table 1).

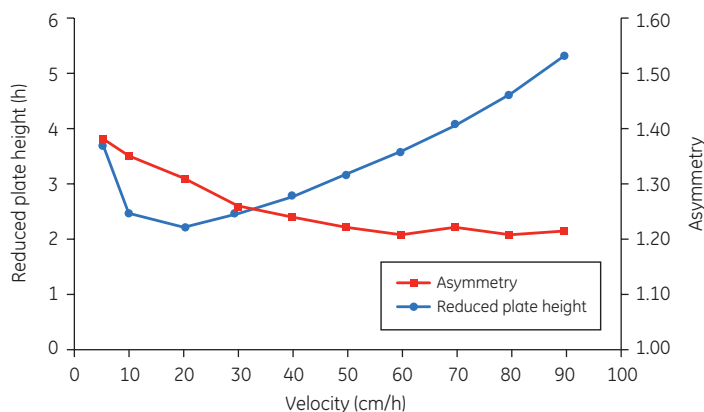


Fig 3. Reduced plate height and asymmetry at different velocities tested on a packed bed. At optimal test velocity (20 cm/h), reduced plate height is 2.3, while at 80 cm/h it is 100% larger.

Table 1. Suitable test velocities for selected media

Particle size (μm)	Example media	Velocity (cm/h)
15	SOURCE™ 15	60
30	SOURCE 30	60
35	Sepharose High Performance, Superdex™	30
50	Sephacryl™	20
75-100	Sepharose Fast Flow, Capto™, MabSelect™	20
200	Sepharose Big Beads	10

Sample volume

Efficiency testing by means of a pulse test is typically based on a sample volume of 1% of the column volume (V_c). Deviations from this rule of thumb may be required due to limitations in available hardware or to enhance detection capabilities of the tracer substance. It should be noted, however, that an increase in sample volume for a pulse test may cause a significant broadening of the peak, yielding an unfavorable plate number for a well-packed column. This is especially valid for chromatography media based on small particle diameters (1). Due to the broadening peak, the peak symmetry might improve. This is equivalent to the impact of increasing test velocity (see Fig 3).

Hold-up volume in test systems

The external volume (V_{ext}) should be kept as low as possible. This volume is defined as the upstream volume (between the point of sample application and the column) plus the downstream volume (from column to the point of tracer detection). An unfavorable set-up of a test system will influence the measured column efficiency similar to the impact of enlarged sample volume (Fig 4). Tubing used for testing should therefore be kept as short as possible with a minimal inner diameter without creating an excessive pressure drop.

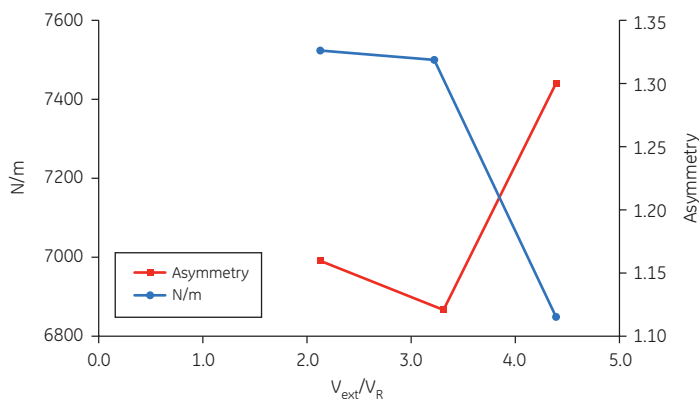


Fig 4. A graphical illustration of the effect of changing the external volume. The external volume was changed from 2.0% to 4.5%. As seen in the graph, small changes in external volume can produce a large effect on the efficiency test result.

When a suitable optimized set-up for efficiency testing cannot be implemented, acceptance criteria have to be adjusted accordingly. This could be the case, for example, when forced to run an efficiency test at low liquid velocity in a system designed for high throughput processing.

As a general rule of thumb, a set-up for efficiency testing of typical columns used in bioprocess chromatography should have an external volume smaller than 5% of the column volume and preferably less than 3% if optimal column efficiency is required. It is strongly recommended to determine the external volume in the system before testing the bed efficiency. Also, a record of the retention volume in an efficiency test should be kept in order to compare retention volume to the nominal retention volume of the packed bed and column only. The retention volume for an inert tracer substance is calculated from the bed voidage ϵ_b , intra-particle porosity ϵ_p , and size exclusion coefficient K_e ($K_e = 1$ for small molecule tracer substance):

$$V_R/V_C = \epsilon_b + (1-\epsilon_b) \epsilon_p K_e$$

If the retention volume is significantly larger than expected, attention should be given to possible interactions between the tracer and the chromatography medium, as well as the holdup volume of the test system external to the column. When the test set-up has been decided and a column has been packed, it is recommended to run three successive tests to measure the repeatability of the method.

Transition analysis

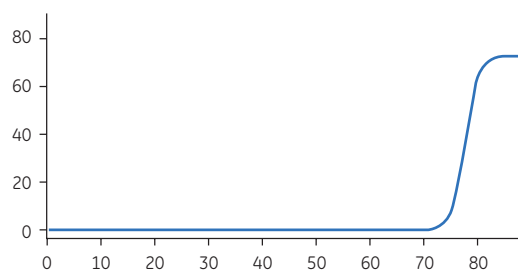
Instead of running a pulse test, a step function of tracer substance can be applied to the column. The resulting breakthrough curve can then be analyzed to derive column efficiency (Fig 5).

The mathematical relationship between pulse function $E(t)$ and the residence time distribution defined by a step function $F(t)$ is

$$E(t) = \frac{dF(t)}{dt}$$

The differentiate function in UNICORN™ evaluation software can be utilized for this operation. As an example, the pulse function $E(t)$ has been plotted below for the step function shown. In a second step, the calculated pulse function can be analyzed as discussed in Figure 1.

Experimental data: Step function



Calculated data: Pulse function

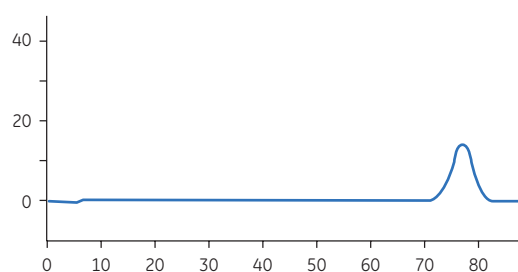


Fig 5. Evaluation of column efficiency for a step function obtained in transition analysis.

Transition analysis may be employed as a method for column qualification due to restrictions in available hardware. In addition, transition analysis can also be used as a tool for online column efficiency monitoring during processing (for example, when following step changes in conductivity). However, consideration should be given to parameters and prerequisites for robust column efficiency monitoring (as outlined above) such as tracer inertness, stability of fluid density and viscosity, and especially fluid velocity. As a consequence, efficiency data obtained by online process monitoring typically differs from data obtained during initial column qualification by the pulse method.

Summary

Specifications for qualification of optimal column efficiency may be defined as:

Reduced plate height $h \leq 3$

Asymmetry factor $0.8 < A_s < 1.8$

These specifications rely on the use of optimized test conditions and parameters including:

- An inert tracer substance to avoid interaction with the medium
- Optimal liquid velocity enabling highest theoretical efficiency
- Low external volume
- A low sample volume in the pulse test (1% of V_c)

Specifications for column qualification as well as test procedures should always be reviewed with regard to specific application needs as well as practical constraints that may not allow for application of optimal test conditions. The specification for reduced plate height and asymmetry may very well be different for different purification steps in the process.

In addition to the efficiency test described in this application note, additional parameters could be used to track the process (e.g., pressure of the bed and retention volume of the product).

References

1. Hagel, L. *et al. Handbook of process chromatography 2nd ed.*, John Wiley and Sons, Inc., New York (2008).

Recommendations for experimental procedures

System preparation

- System and tubing should be primed.
- Air that may have collected in the tubing and distribution system during packing, storage, or transportation should be removed.
- The column should be equilibrated in the eluent with at least $1.5 V_c$. If equilibrating the column into or from salt, the outlet conductivity should be equal to the inlet conductivity. The equilibration should be run in the same direction as the test. The test can be carried out either in the upflow or the downflow direction.

Running the pulse efficiency test

A predetermined amount of sample (1% of V_c) is applied to the column, preferably by using a sample loop, a superloop, or a sample pump. If none of these devices is available for sample dispensing, the test may be run by priming the system with sample in a first step while running the column in bypass mode. After application of the desired sample volume to the column, the column is switched to bypass mode to exchange the sample to eluent before finally running the actual pulse test.

Step-by-step instructions for efficiency testing without having a loop or sample pump available

1. Place the eluent on one system inlet and sample on another. The sample can be placed on another inlet, on pump A, or on pump B if the system has gradient capabilities.

2. Bypass the column, filter, and air trap and open the sample inlet. Run sample until a stable reading is achieved in the UV (or conductivity) sensor. In order to optimize the sample volume that needs to be prepared, this required volume should preferably be measured before running the test automatically.
3. Once the system is fully primed with sample, place the column inline and run the sample volume to be injected. Make sure the sample is injected at the same flow rate as the elution step will be run.

Note: If a step function is to be applied for the purpose of a transition analysis, the sample is applied until complete breakthrough is achieved.

4. For the pulse test, bypass the column, shift the inlet from sample to eluent, and increase the flow rate. Continue until all sample is flushed from the system.
5. Lower the flow rate to the test flow rate and when that is reached, place the column inline, using the same flow direction as when sample was applied.
6. Note that the sample injection mark on the chromatogram should be in the center of the injection, since the true retention (elution) volume is from mid-injection to peak apex.
7. Run the elution until the entire peak has exited the column.

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