Chromatography Column Performance and Data Analysis Success Guide

Hints and Tips for Better Purifications

BIO-RAD

This Chromatography Success Guide provides practical advice on preparative chromatography and protein purification.

Read on to discover key tips, secrets, and recommended resources every chromatographer should know about.

Data Organization: Keep It Tidy

Data organization — before any type of analysis, it's a good idea to make sure that all relevant data are centralized in a single folder and that all files are named in a consistent manner to avoid confusion.

Data display — focus on the data you need! There may be multiple signal traces or curves associated with your run, and in most instances you will require only a subset. For many analyses, displaying only the UV trace along with the gradient trace is sufficient. Hide or remove all unused traces.

What’s in Your Peak?

Peak area — the area under the curve of the UV trace to its baseline. This is often correlated with the amount of protein.

Peak retention time — the time it takes for a peak to come off your column. This can be measured from the start of your run to the apex of your peak of interest. However, the most common method is to measure from the injection of the sample to the apex of the peak.

Retention volume — the volume of liquid needed to pass through your column to elute the peak from your column. The most common method is to measure the volume from the injection of the sample to the apex of the peak. You may also measure the volume from the start of the run to the apex of the peak.

Peak height — the distance from the bottom or baseline of the peak to its apex. The bottom of the peak is defined by either a zero absorbance value or a calculated baseline for increased accuracy.

Relative area — the percentage of the entire calculated peak area represented by a single peak area. This is used to determine yield, purity, or level of contaminants.

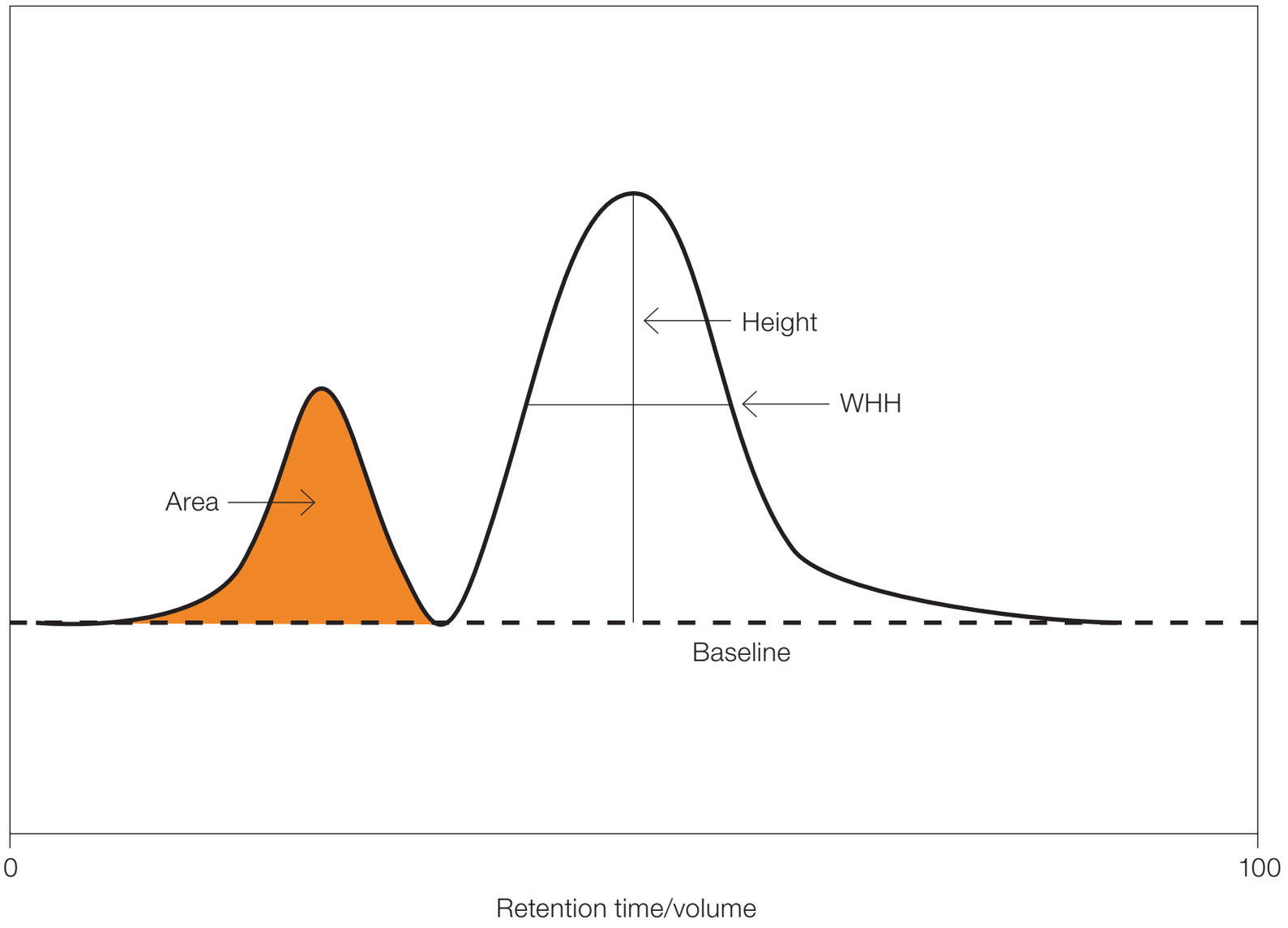
Injection point — the time at which the sample is injected onto the column. For example, when using a sample loop, this is the point at which the loop is placed inline. This is often used as the zero time point for measuring peak retention time.

Delay volume — the added volume of liquid present due to the length of tubing between the detector and the head of the fraction collector. This value is important because it is used to ensure that the fraction marks line up properly relative to the peaks.

Peak resolution — although there are some mathematical definitions for this in chromatography, it is the relative distance between the apexes of two neighboring peaks.

Width at half height (WHH) — a measure of the separation efficiency. The lower the value, the thinner the peak and, therefore, the more efficient the column. In general, columns with smaller beads will have lower values for WHH. It is equal to the distance between the peak boundaries at half the peak height.

Anatomy of the peak



Peak asymmetry — the asymmetry factor (AS), defined as the distance from the center line of the peak to the back slope divided by the distance from the center line of the peak to the front slope.

In general, the AS is a measure of the quality of column packing. Well-packed columns will have an AS of close to 1. All measurements are made at 10% of the maximum peak height.

Purity (using peak area) — sample purity can be estimated directly from the chromatogram by comparing the peak areas for the peak of interest vs. all other peaks in the chromatogram. This is usually expressed as the percentage of the total peak area represented by the peak area of interest. This also assumes that the peak of interest is pure and contains all of the target protein.

Purity measured by peak area will only take into account purity levels against proteins that absorb at the wavelength used for detection.

Shoulder — peaks exhibit shoulders when proteins elute from the column at or near the same time, hence the smaller peak partially merges with the larger one and becomes a “shoulder”. Methods should be written and optimized to minimize the amount of shouldering for proteins of interest to ensure maximum purity of the peak.

Uh-Oh, There's DNA in My Protein!

Use a multiwave detector — this will add flexibility to your chromatography system, enabling the simultaneous monitoring at up to four wavelengths for the detection of proteins, peptides, nucleic acids, and chromophores such as hemoglobin in complex biological mixtures.

The ratio of average absorbance at 280 nm and 260 nm (or 255 nm) can be used to determine the purity of your protein from DNA contaminants. Some chromatography software programs will allow you to calculate this value for each fraction, allowing you to select fractions containing the least amount of impurities.

How Is My Column Doing?

Column performance — the performance of a chromatography column may be determined by measuring the number of theoretical plates (N). These theoretical plates are hypothetical stages where substances come into equilibrium.

The greater the number of theoretical plates for a column, the more efficient the separation.

HETP — in order to normalize N across columns of different sizes, divide the length of the column by N. This provides the height equivalent to theoretical plate (HETP) value. The lower your HETP the more efficient the separation. The HETP can be measured over time to monitor column performance.

An increasing HETP value indicates loss of column performance.

To perform an HETP analysis to measure your column's performance, use a sample that does not interact with the column material, such as acetone or concentrated buffer. Inject the sample via the sample loop and elute under isocratic conditions. After the run, calculate the column efficiency in terms of HETP and the peak asymmetry.

Backpressure — Increasing backpressure under the same column conditions could indicate fouling, which may lower performance.

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