CHROMATOGRAPHY

The Basics of HPLC/UHPLC and LC Columns Training Guide

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Questions and Answers

What Is Chromatography?

Chromatography is a general term for a laboratory technique used to separate components in a sample. Examples of chromatography include liquid chromatography (LC) and gas chromatography (GC). Chromatography consists of two phases: a mobile phase and a stationary phase. The mobile phase in LC is a liquid, whilst in GC it is an inert gas (typically hydrogen or helium). The stationary phase is essentially a column. In LC, the column is a packed tube, whereas in GC, the column could be a packed tube, but more commonly is a material with a coating on the Inside (known as a capillary GC column). LC is the most widely used analytical technique in the world because its only requirement is that the sample dissolves in a liquid. The remainder of this training guide will focus on LC and LC columns.

In LC, the sample is dissolved in a liquid or the mobile phase which carries it through a stationary phase. The various components of the sample travel through the column at different speeds due to their interactions between the mobile and stationary phases, resulting in the components separating from one another. The specific chemistry of the stationary phase and mobile phase used determines how the components of the mixture interact, and therefore which ones travel more quickly or slowly. The different travel times are referred to as the components' retention time. The mobile and stationary phases selected will differ depending on the analysis. The goal is to separate all components of interest.

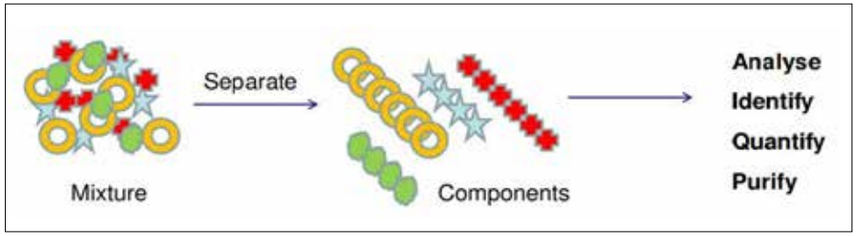

Figure 1. Generic schematic demonstrating separation of a mixture into its components using chromatographic techniques, as well as what is commonly carried out post separation.

What Is a Column?

In chromatography, the column is responsible for the separation of the sample components within a mixture. HPLC columns typically consist of a tube (typically ≤ 25 cm in length), packing materials and end fittings.

Figure 2. LC columns of varying size.

What Is the Tube Made Of?

Most HPLC columns (Figure 2) are made of high-quality passivated stainless steel for highest pressure resistance, with highly polished, mirrored finished internal bores. See the next question for details on what is Inside the column.

What Is Inside a Column and What Is the Stationary Phase?

The Inside of an LC column is packed with the stationary phase (it is also referred to as adsorbent or packing material) under high pressure. This stationary phase interacts with and retains the compounds.

The most common packing material is high purity porous silica particles which can either be unmodified, or chemically modified to create specific column chemistries. Some common modifications include the addition of hydrocarbons in various chain lengths (e.g. C18, C8 and phenyl). Figure 3 demonstrates an example of a C8 column, where the column is packed with silica particles which have been chemically modified by bonding C8 hydrocarbon chains (8 carbon atoms).

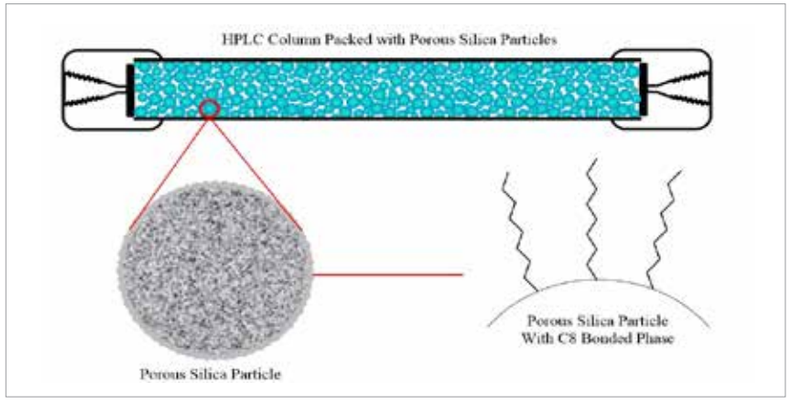

Figure 3. Schematic of a traditional HPLC column packed with porous silica particles. In this example, the silica particles have been chemically modified by attaching a C8 hydrocarbon chain (now a C8 column).

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What Is a Mobile Phase?

In LC, the mobile phase is a liquid (e.g. buffers, water, organics) which interacts with and carries the components through the column. The mobile phase differs depending on the application and variations can include changes in type of organic solvent and its percentage, concentration and type of buffer, and pH.

An example of a simple mobile phase is 80% Methanol/20% Water.

What Is a Chromatogram?

A chromatogram is an XY graph which plots detector response against time (Figure 4).

From a chromatogram you can determine:

1. How many components are in a sample (number of peaks).
2. The identity of each component (retention time in comparison with a standard).
3. The amount of each component (peak area in comparison with a standard).

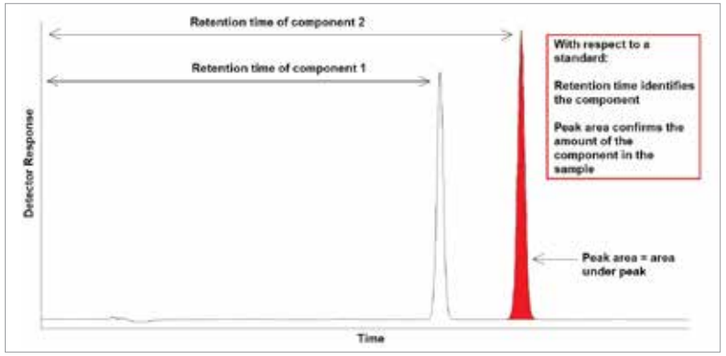

Figure 4. Example chromatogram of a sample containing two components.

What Is a Chromatograph?

A chromatograph is an instrument (e.g. HPLC system or UHPLC system) for performing chromatographic separations and producing chromatograms. A basic chromatograph consists of a pump, autosampler or manual injector, and a detector.

What Is HPLC?

High performance liquid chromatography (HPLC) is used to separate, identify, and quantify components in a mixture. Figure 5 provides a schematic of the components of a typical HPLC system. The sample of interest (liquid sample) is injected into a continuously flowing stream of liquid solvent (the mobile phase), which is pumped through a stainless-steel column packed with a separation medium (the stationary phase). In the column, the mixture is separated into its constituent components through different interactions with the stationary phase. As a result, the components move through the stationary phase at different rates. When the components emerge from the column, they are carried to a detector where a physical property of the compounds is measured (e.g. absorption of light for UV detection). Each response is graphed over time, resulting in a chromatogram (Figure 6).

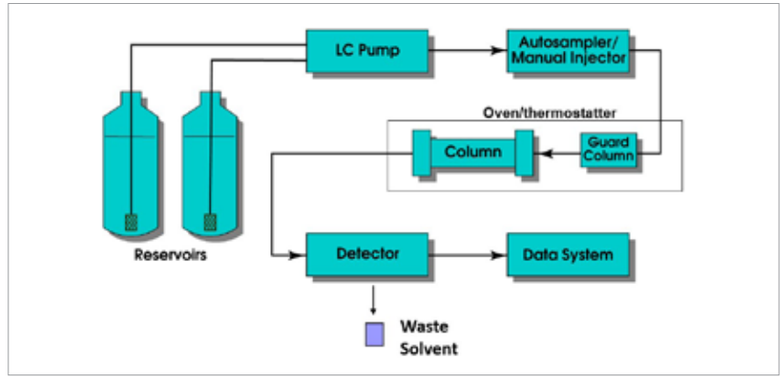

Figure 5. Schematic of a chromatograph/HPLC system showing the components of the system and the direction of mobile phase flow.

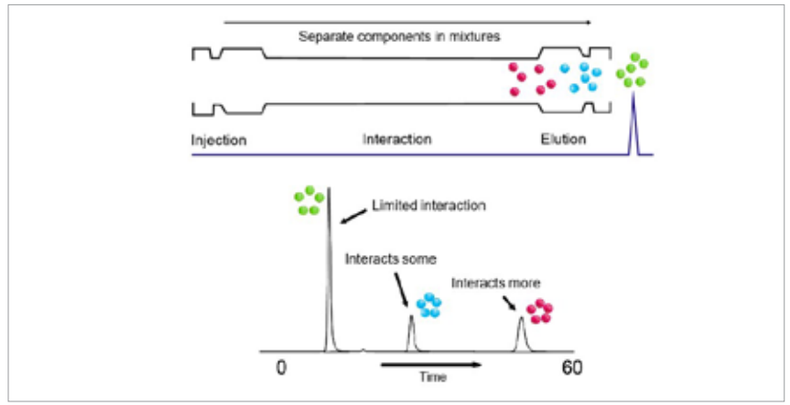

Figure 6. Schematic of a mixture being separated into its constituent components in an LC column, being detected and thus producing a chromatogram.

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What Is the Difference between HPLC and UHPLC?

When the mobile phase is pumped through the stationary phase, pressure is created (due to resistance of the flowing liquid against the packing material). HPLC (high-performance or high-pressure liquid chromatography) and UHPLC (ultra-high-performance liquid chromatography) are both liquid chromatography techniques used to separate different compounds found in a mixture. However, UHPLC can operate at higher pressures (typically greater than 6,000 psi (414 bar) to allow for analysis using columns with smaller particle sizes (< 2 µm) or longer SPP columns. UHPLC improves analyte (compound of interest) resolution and sensitivity, shortens run times, and improves productivity. It also reduces solvent consumption which reduces cost and the environmental impact. However, UHPLC requires dedicated hardware with lower dispersion (smaller volume flow cells, narrower and shorter connection tubing, smaller sample loops).

Our LC 300 HPLC operates up to 10,000 psi (690 bar), whilst our LC 300 UHPLC operates up to 18,000 psi (1240 bar).

What Is the Difference between Analytical and Preparative Chromatography?

Chromatography can be analytical, semi-preparative or preparative. In analytical chromatography, the purpose is to separate out each of the components of the sample and analyze specific components in detail to gather information (e.g. identification and quantitation). Typical analytical columns have internal diameters I.D. of ≤ 4.6 mm.

The purpose of semi-preparative and preparative chromatography, on the other hand, is the separation and collection of a purified component on a much larger scale (much larger quantities of sample and larger columns). Typical semi-preparative columns are 10 mm ID and preparative sized columns are ≥ 20 mm I.D.

In analytical chromatography, the mobile phase typically goes to waste after going through the detector. In preparative chromatography, the mobile phase is often passed on to a fraction collector to collect large quantities of the component of interest. It should be noted that sometimes in analytical chromatography, a component will also be collected.

PerkinElmer does not sell preparative chromatography instruments, but all of our LC columns are fully scalable from analytical to preparative dimensions and can be used on any manufacturers LC systems.

What Is Meant by Polar, Non-Polar and Ionic Compound?

Polar compounds exhibit partial charges (i.e., positive or negative) in their structure that may interact with other partial charges either in the stationary or mobile phase. Therefore, the partially positive end of one polar molecule is attracted towards the partially negative end of another molecule. Think of mixing water and olive oil. The two liquids do not like to interact and will be driven to separate into layers. The differences in the amount of charge exhibited by the compound itself (more or less polar) as well as shifting polarity of the mobile phase itself will control how strongly the compound is attracted to the stationary phase and therefore the retention time of the compound. The same concept can be applied to non-polar compounds.

Ionic compounds exhibit much stronger charge and therefore the interaction is strong ionic interaction.

Figure 7 demonstrates how polarity differs for different types of compounds in samples.

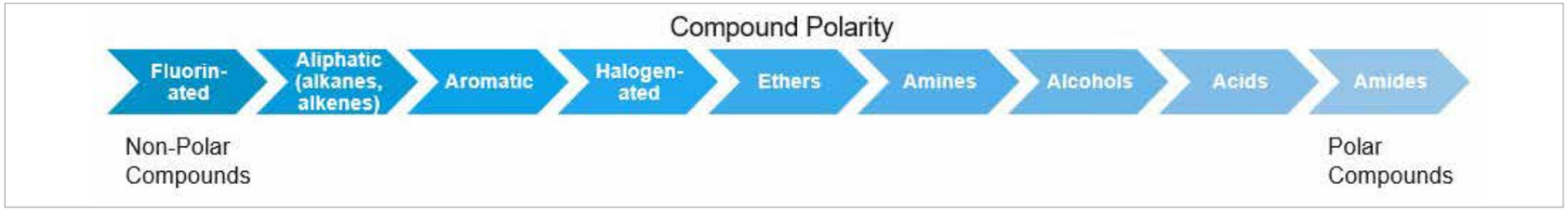

Figure 7. Examples of types of compounds which may be found in samples and how their polarity differs with respect to one another.

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What Are the Main Types of Separation Modes in HPLC/UHPLC?

The two most common variants of HPLC are 'NP' and 'RP' HPLC. Other modes include ion-exchange and size exclusion. RP-HPLC is the mostly widely used form of HPLC/UHPLC.

In NP-HPLC, the stationary phase is polar (e.g. unmodified silica), whilst the mobile phase is non-polar (e.g. hexane). In this mode, non-polar compounds in the sample mixture will travel more quickly through the column while polar compounds will interact more with the polar stationary phase (like attracts like).

In RP-HPLC, the reverse occurs. The stationary phase is non-polar or less polar than the more polar mobile phase. The stationary phase is a chemically modified silica (e.g. bonding hydrocarbons like C18 to the silica) to make it non-polar or less polar. A polar solvent is then used as the mobile phase such as a mixture of water and methanol or water and acetonitrile. In this mode, polar compounds in the sample will travel through the column more quickly because they are not attracted to the non-polar stationary phase. The interaction is a weak hydrophobic interaction. As the number of carbon atoms increase, the compound is typically more non-polar, and will elute later (an example is shown in Figure 8). Figure 9 demonstrates how polarity differs for different types of solvents in mobile phases.

Ionic compounds are analyzed by ion exchange chromatography rather than RP or NP chromatography. Typically, the aqueous component of the mobile phase will be a buffer/salt (e.g. phosphate or acetate).

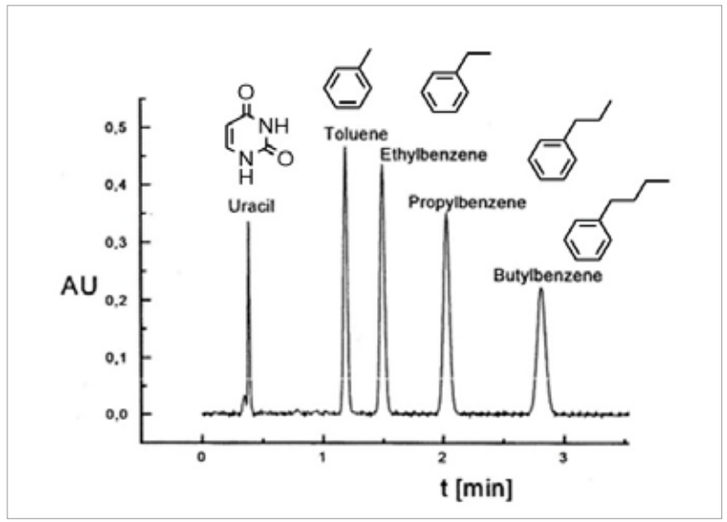

Figure 8. In reverse phase, as the number of carbon atoms increase, the compound becomes more non-polar and will elute later. This example shows analysis of a test mix containing five compounds using a C18 column. Uracil is a very polar compound which has no retention on a C18 column and therefore elutes first. Toluene, ethylbenzene, propylbenzene and butylbenzene have an incremental increase in the number of carbons (becoming more non-polar) and therefore eluting in this order.[1]

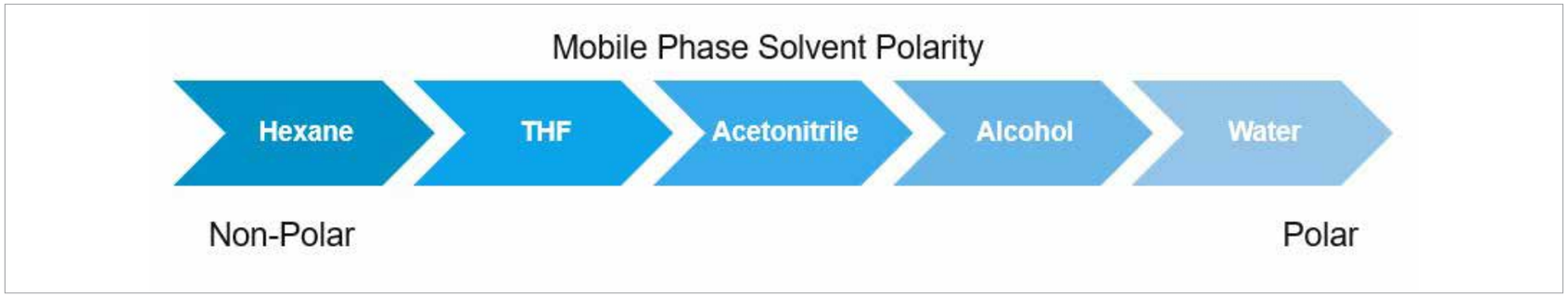

Figure 9. Examples of solvents used in mobile phases and how their polarity differs with respect to one another.

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How Do You Choose a Stationary Phase/Bonding Chemistry?

The choice of stationary phase/bonding chemistry is application dependant. Many analytical methods have well established chemistries that have been used for years and provide reliable and reproducible results. These chemistries are not always the ideal separation method for the component of interest, but adherence to regulations often require these chemistries and changing regulated methods can be a burden to laboratories. If working on a new application, chemists usually use a column they already have, use ones which worked previously for a similar analyte, or look for applications from literature and vendors using similar analytes.

If there is limited literature or knowledge about a particular analysis, or the sample contains unknown analytes, a toolkit approach is a highly efficient method for determining the most appropriate column for the application. This approach involves firstly determining what the sample is soluble in to determine which separation technique is needed (e.g. RP, NP). Once this has been determined, the next step is screening multiple columns (the toolkit approach) which have very different bonding chemistries (e.g. C18 and PFP) to provide alternative modes of interaction to help achieve the desired separation more quickly.

What Is a USP Column Code?

LC columns are typically grouped into categories based on their stationary phases, with each category being given an 'L' code (e.g. L1, L7).

These 'L' codes are often referred to as 'USP' column codes which come from United States Pharmacopeial convention. Most column phases have a USP code which describes a general grouping for materials. As an example, all C18 columns have the same USP column code (L1). USP describes 'L1' as "octadecyl silane chemically bonded to porous silica or ceramic micro-particles, 1.5 to 10 µm in diameter, or a monolithic rod". It is important to note that a C18 column may be referred to 'ODS' which simply means 'octadecylsilyl' (C18 is the same as ODS).

USP monographs specify the column required in the method using the USP column code notation.

If a Manufacturer Sells a Certain Column Chemistry, Will It Give the Same Results as Another Column Manufacturer (e.g. PerkinElmer Epic C18 vs. Waters Symmetry C18)?

Using C18 columns as an example, these columns have a broad applicability from pharmaceuticals to food and environmental analyses. However, not all C18 columns are alike and often will produce slightly different results. Simply swapping a C18 column from one manufacturer to another can result in differences in retention time, resolution and even selectivity. Differences can arise due to variations in the way the stationary phase is manufactured, packing quality, particle size distribution, and silica purity, to name a few. Slight adjustments to the mobile phase will usually result in an acceptable separation.

Our range of clone columns offer a cost-effective comparable alternative to many older legacy column brands (e.g. PerkinElmer Harmony™ is an equivalent to Waters Symmetry® HPLC column).

What Do LC Column Dimensions, such as 150 x 4.6 mm, 5 µm, Refer to?

HPLC columns are manufactured in a variety of lengths, internal diameter (I.D.) and particle size. In the example above, 150 mm is the length of the column, 4.6 mm is the I.D. of the column, and 5 µm is the size of the particles within the column. These dimensions are one of the most common for routine analysis.

Column dimensions will affect the efficiency, sensitivity, and speed of the analysis. The choice of the LC column is dependent on the chromatographic application and customer requirements (e.g. a customer may require good resolution but is not concerned about speed of analysis). Column dimensions can be altered to improve the separation based on the customer's requirements (e.g. increased efficiency, sensitivity, or speed). This is the reason for offering columns with the same chemistry but with different lengths, I.D.s and particle size. However, there is a trade-off. As an example, increasing the length of a column, will increase the ability to separate the peaks, but retention times will be longer, and the pressure will be higher.

Typical LC analytical column dimensions are shown below.

Column Length: 50 mm – 250 mm*

Column I.D.: 2.1 mm – 4.6 mm (fixed-bed preparative columns are often between 10 - 75 mm I.D.)

Particle Size: < 2 µm – 5 µm* (preparative columns use > 5 µm)

** Some analytical methods may use 300 mm length columns, and 10 µm particle sizes.*

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What Is Meant by Column Pore Size?

LC columns commonly contain a packed bed of silica particles with each particle having 'pores'. The column pore size refers to the average diameter of these pores and determines whether a molecule can diffuse into and out of the particles (Figure 10). The pore size of the packing is therefore important to ensure that the molecules in the sample can 'fit' into the pores to interact properly with the stationary phase.

Columns are typically separated into two types:

- **Small-pore** particle columns typically have pore sizes ranging from 60-150 Å (with majority being 80-120 Å) and are used for the analysis of small molecules. Pore diameter is not usually a primary consideration when selecting a column for small-molecule analysis.
- **Wide-pore** particle columns have pore sizes > 300 Å and are used for the analysis of large molecules such as large peptides and proteins.

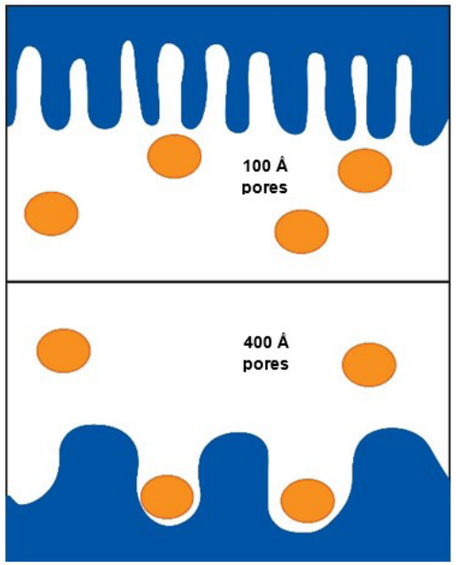

Figure 10. Representation of small pore particles (~ 100 Å) vs. wide pore particles (> 300 Å). Smaller pores do not allow most proteins to enter the pores, which limits interaction.

What Is a Guard Column and Why Are They Used?

Guard columns are installed between the injector and the LC column as an easy and inexpensive way of protecting and extending the lifetime of the more expensive analytical LC column. Guard columns will prevent any particulate contaminants and highly absorptive compounds in the sample from entering and contaminating the column.

The guard column should have the same type of packing material as the LC column (e.g. C18 column and C18 guard). Often these guard columns are available in a cartridge format. They fit Inside a holder (Figure 11) and are replaced regularly or at the first sign of any change in back pressure of the system or in chromatographic performance. The advantage of a cartridge design is the holder may be reused over and over, saving on the expense of end fittings as well as environmental impact.

Figure 11. Guard column/cartridge and holder.

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Where Does a Column Fit In Our LC 300?

All versions of the LC 300 Autosampler have an integrated column oven. This onboard oven is a heat only oven and can accommodate columns up to 150 mm in length.
An external Peltier column oven is also available which can be used for columns up to 300 mm in length and has an option for a column switching valve.

Do PerkinElmer Columns Work on All Instrumentation?

Yes, all PerkinElmer columns will work on all HPLC/UHPLC instrumentation (PerkinElmer and competitive suppliers).

More Information

Liquid Chromatography Columns Catalog

References

1. B. Bidlingmaier, K.K. Unger & N.von Doehren, Comparative study on the column performance of microparticulate 5-µm C18-bonded and monolithic C18-bonded reversed-phase columns in high-performance liquid chromatography, Journal of Chromatography A, 1999, 832, 11-16.

COMMON DEFINITIONS

EFFICIENCY	A measure of column performance and is also termed number of theoretical plates. The greater the number of theoretical plates, the greater the chances the column will resolve all peaks. Higher efficiencies provide sharper and narrower peaks, resulting in higher sensitivity and improvements in resolution.
RESOLUTION	A measure of the separation of two peaks.
RETENTION TIME	The time taken for a component to pass through a chromatography column and be detected (it is the time from injection to detection).
REVERSE PHASE	Most common form of HPLC/UHPLC. Typically uses a C18 column with a water/buffer and methanol/acetonitrile mobile phase.
SELECTIVITY	A measure of the ability of a chromatographic system to separate two components from each other.
SENSITIVITY	Ability to detect a target component. Usually expressed as the lowest amount of a component that can be detected.

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