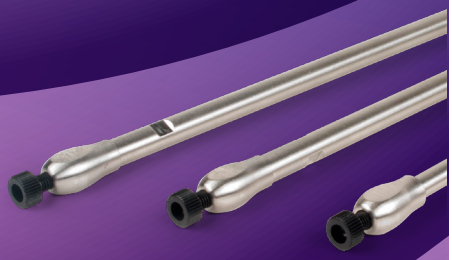
KINETEX® Core-Shell Technology

AXIA™ Preparative Columns

explore LUNA® OMEGA

LUX® Chiral LC Columns



phenomenex®

Frequently Asked Questions
for UHPLC/HPLC

General LC

Why run a gradient from 5-95 % organic solvent, instead of 0-100 %?

When running a gradient, it is important to remember that the total time for each run includes both the run time, and the necessary re-equilibration before the next run.

When running from 0-100 % organic solvent the re-equilibration step is far longer than for 5-95 % such that the majority of users sacrifice the small restriction in terms of method flexibility for a much larger gain in productivity.

There is speculation on the mechanistic reason behind the lengthy equilibration time required for methods utilizing 0 % aqueous and/or 100 % organic solvent in the gradient. Phase wetting/de-wetting and phase collapse have been proposed causes.

Either way, in practice, variations in retention can be observed with alkyl phases using 100 % aqueous conditions. If necessary for retention of certain highly polar compounds, we recommend columns stable under such conditions, such as from our Synergi brand of **HPLC** columns.

The pressure in my HPLC system is low and erratic, and the system keeps shutting down. How do I fix this problem?

First, check for leaks. If the system is in regular use, connections that are regularly made and broken (e.g. columns, guard columns, etc.) are potentially the weak link. If a leak is suspected, disconnect the tubing from the leaking fitting to ensure that the end of the tubing and the connecting nut and ferrule, or finger-tight fitting, are free from damage. Then reconnect; don't be tempted to simply over-tighten as this will normally cause irreparable damage.

If there is no leak, then the most likely cause of the problem is air within the system. Check that your mobile phase is adequately degassed when prepared or ensure that your online degasser is switched on and is functioning correctly.

Should I use TFA or Formic Acid for my peptide separation on Aeris PEPTIDE?

TFA(trifluoroacetic acid) is generally the preferred mobile phase buffer additive for reversed phase **separation of peptides** and protein. While some researchers prefer to use formic acid buffer where MS detection is used, the preference for TFA in the mobile phase relates to the fact that TFA is a weak ion-pairing buffer.

I need to start work with a new ion-pairing method this morning, how can I quickly equilibrate my column for use?

Under planned circumstances, equilibrating a new column for use in an ion-paired method would best be achieved overnight before the first run. If this is not possible, then the following procedure can be adopted:

General LC (cont'd)

1. Make up a solution of the ion-pair which is tenfold greater in concentration than the concentration in the mobile phase for the method. Note pH of mobile phase and column pH limitations and adjust appropriately.

2. Make large volume (50-100 µL) injections of this solution 5-6 times, allowing 1-2 minutes between each injection.

3. Continue to run the method mobile phase through the column until a stable phase line is achieved.

4. Check equilibration of the column by making replicate injections of a standard solution, the column is fully equilibrated when retention times and peak areas are consistent between injections.

What is the best way to remove ion-pairing reagents from my HPLC column?

Washing the column with a strong organic solvent, such as acetonitrile, should remove a moderate amount of the ion-pair before column storage.

However, because **ion-pairing reagents** can alter column selectivity, it is advisable to dedicate columns to ion-pairing methods to prevent problems with reproducibility.

If you have any specific questions regarding methods using ion-pairing reagents or if you would like to try alternative methods or column chemistries so as to avoid ion-pair, please **contact us**.

My column lifetime is consistently short, what is the issue here?

One must consider method parameters when evaluating column lifetime.

Running close to the pH limitations of the column may lead to accelerated column failure. High pH may accelerate silica dissolution while low pH may hydrolyze stationary phase.

Running at relatively high backpressures will accelerate void formation, as silica dissolution invariably occurs over time.

What changes are allowed to USP / EP gradient HPLC methods?

When using a gradient method, no change may be made to particle size or column dimensions. They must be the same as those directed in the monograph.

What are the most common causes for baseline drift with RI detectors?

Changes in temperature and pressure are one of the most common causes of irregular baseline drift with RI (refractive index) detectors.

If the column temperature is more than 20 °C above ambient, consider using a secondary heat exchanger to reduce the temperature of the mobile phase that exits the column to as close to the flow cell temperature as possible and also minimize any temperature fluctuations.

If pressure is the suspected problem, it could be a problem with the pumps, e.g. pump seals need changing. Turn off the pumps and see if the fluctuations are still observed.

Finally, another cause could actually be the lamp itself, usually tungsten or LED with RI detectors. If the lamp is weak, this can cause baseline fluctuations as well.

How should I store my HPLC columns?

In general, storing columns with buffer is not recommended.

If the column is stored at acidic pH for more than a week there is potential for ligand hydrolysis and if stored at basic pH the silica can begin to dissolve. It is also recommended to store with >50 % organic solvent (e.g. acetonitrile), to reduce silica solubility and inhibit microbial growth.

How can I calculate gradient dwell volume on my HPLC?

The simple solution would be to use an UV-absorbing solvent as the mobile phase B (e.g. acetone), remove the column, and run a method similar to below:

Mobile Phase A: HPLC Grade Water
Mobile Phase B: Acetone
Flow Rate: 2 mL/min
Detection: 265 nm
Gradient Program

Time (min)	%A	%B
0	100	0
10	0	100
15	0	100

The gradient dwell volume can be calculated based upon the time delay where the gradient starts. If the UV signal increase starts at 12 minutes, the gradient dwell volume would be 4 mL.

A common practice is to have an isocratic portion (e.g. hold 5 % B for 2 minutes) at the beginning of the run to compensate for any differences in gradient dwell volumes between systems.

General LC (cont'd)

When I run a gradient LC on one instrument, the results are acceptable. However, when transferring them to another system, there are differences in retention. What could be the cause of this?

Differences in gradient dwell volume or the volume between the gradient mixer and the head of the column may result in shifts in retention.

The gradient itself should also be investigated. Although gradient systems on most modern HPLC/UHPLC systems operate with a reasonable amount of precision, steep gradients with disproportionate amounts of Mobile Phase A and B may result in retention time discrepancies.

What solvents can be used as a mobile phase under HILIC conditions?

The organic solvent type can be varied to change retention and separation selectivity, much like reversed phase separations. Solvent strength (from weakest to strongest) for HILIC generally is tetrahydrofuran < acetonitrile < isopropanol < ethanol < methanol < water, where water is the strongest elution solvent. Note that to further increase retention in HILIC, using another polar solvent such as methanol or isopropanol with water is sometimes effective. As such, acetonitrile is the preferred weak solvent in HILIC mode.

For optimum column efficiency and reproducibility, buffers in the range of 10 - 20 mM concentration or additives in the 0.5 % range are used in the mobile phase.

Phosphate buffers are not recommended because of their poor solubility in high organic mobile phases, in addition to being incompatible with MS detection, where HILIC is commonly used.

I am seeing a negative peak in my reversed phase chromatogram. I am using a UV detector. What is causing this?

Any difference in the mobile phase and sample will cause a peak; i.e. regardless any change in mobile phase composition will cause a response. So, if the absorbance of a solute is less than that of mobile phase, this can cause a negative peak. As such, this method may require a mobile phase with a lower UV absorbance.

What are the advantages of analyzing my basic API at high pH?

At high pH, basic compounds will be deprotonated, making them less polar/more hydrophobic. As such, the basic compound will be better able to interact with a hydrophobic stationary phase such as that provided in a C18 column. This results in longer retention times.

Secondary interactions are reduced, improving peak shape, as an additional benefit.

Finally, increased retention of basic compounds typically leads to elution in higher organic, which may help ionization in LC-MS applications.

Basic compounds therefore retain longer, with better peak shape, when you work at high pH. **Kinetex EVO** columns were specifically designed to help analysts utilize pH as a tool for method development.

How can I maintain high efficiency during scale up from analysis to purification?

Using **AXIA** packed preparative columns gives you up to 30 % higher efficiencies than traditional slurry packed columns.

What tubing and fittings can I use to connect my AXIA preparative column to my HPLC system?

21.2 mm and 30 mm ID AXIA columns are compatible with 1/16" tubing and 10/32 fittings. 50 mm ID AXIA columns are compatible with 1/8" tubing, although they are shipped with reducing unions allowing for the use of 1/16" tubing and fittings.

Why does the thermal treatment of Luna Omega result in higher efficiency?

The use of temperature greatly reduces the contribution of micropores to the overall measured surface area of Luna Omega. The reduction of micropores allows for molecules to migrate into and out of the pore structure effectively, increasing the efficiency of the separation.

How should I transfer a Lux Chiral column from Normal Phase to Polar Organic or Reversed Phase?

To safely transfer a column from normal phase to polar organic or reversed phase conditions, flush the column with methanol/ethanol (9/1) as a transition solvent.

Why does Phenomenex use DEA over TEA as the primary basic modifier in most of their Lux chiral appliction notes?

Both DEA (diethylamine) and TEA (triethylamine) are widely published as good basic modifiers for improving peak shapes on polysaccharide-type chiral columns. We chose DEA for our initial screening data and have continued with such routinely to maintain consistency. TEA is also just as effective and also commonly used successfully in practice on our **Lux** polysaccharide chiral columns.

Core-Shell & Fully Porous

What are the primary benefits of Kinetex core-shell technology compared to fully porous particles?

Core-shell particles allow chromatographers to achieve UHPLC performance at significantly lower backpressures compared to sub-2 µm fully porous materials. This translates to increased resolution, higher sensitivity, and faster analysis on conventional HPLC systems and UHPLC systems. Kinetex 1.7 µm core-shell particles have been shown to outperform sub-2 µm porous particles by 20 % producing greater resolution and increased sensitivity.

Are there any drawbacks of Kinetex core-shell technology?

Kinetex core-shell technology provides significant performance advantages in the analytical environment it is currently not available in preparative scale chromatography for purification purposes.

How does the lifetime of Kinetex core-shell columns compare to fully porous columns?

The lifetime of any column depends on factors such as the sample, sample preparation/column protection effectiveness, the mobile phase quality, and how well the system is maintained. Chromatographers can expect Kinetex column lifetimes equal to or greater than those achieved on fully porous UHPLC columns. For significantly increased column lifetimes, use **SecurityGuard ULTRA** guard cartridges to protect your Kinetex UHPLC column.

Do I need to revalidate my method if I swap my current column to a Kinetex core-shell column?

You may not need to revalidate your method when switching to Kinetex. Governing bodies such as USP (United States Pharmacopeia), EP (European Pharmacopeia), ICH (International Committee on Harmonization) state that certain changes in column dimension, particle size, and running conditions are allowable without the need for method revalidation. However, most companies have their own guidelines for what determines whether method revalidation is required and should be consulted first.

What is the typical efficiency of Kinetex 5 µm analytical column when compared to analytical columns of the same dimensions packed with fully porous 5 µm stationary phase?

As reported by Gritti et al, efficiency of columns packed with core-shell particles will be significantly higher than fully porous particles of the same diameter (1).

As such, the typical efficiency in plates per meter for Kinetex 5 µm is around 180,000, while for fully porous 5 µm columns with the same dimensions is around 100,000.

How can I determine the void volume of a Kinetex column?

This is best performed experimentally. Multiplying the elution time of an unretained compound by the flow rate will give the actual void volume of the system and column. For example, uracil is commonly used as a void marker in reversed phase separations as it is generally unretained by reversed phase columns. To determine the column void volume alone you would need to subtract the system void volume determined without the column attached.

Alternatively, the void volume can also be estimated by taking ½ of the column volume for an **HPLC/UHPLC** system with minimal void volume. Note this differs from the void volume of a column with fully porous material, which is higher. For example, 60 % of the column volume in the case of a 3 µm Luna material.

Which Kinetex particle size should I use (1.7 or 2.6 µm)?

The efficiency of a 1.7 µm column can improve up to 10-35 % higher than what is obtained with the 2.6 µm, depending primarily on the amount of dwell volume in the system and data acquisition rate. However, the backpressure observed with 1.7 µm Kinetex columns will be more than 2x higher as what is seen with the 2.6 µm columns.

For example, a 100 x 4.6 mm 1.7 µm Kinetex column running at a typical flow rate of 1+ mL/min will likely approach the pressure limits of a conventional HPLC system (300 – 400 bar). So, if using a conventional system without modifications to reduce system dwell volume, it makes more sense to use the 2.6 µm Kinetex material as there will not be large gains in observed efficiency and pressures generated will likely not exceed system limits. However, with an **UHPLC** system, the 1.7 µm material might be preferred because of the higher pressure limits and reduced system dwell volume.

Will my cost per sample decrease by converting my method from a 5 µm fully porous to a Kinetex 2.6 µm core-shell column?

By taking advantage of the up to 3x increase in performance, 2.6 µm columns offer over traditional 5 µm fully porous columns, therefore, you can decrease your cost per sample. A 75 mm length, 2.6 µm Kinetex column has been shown to provide the same resolving power as a 250 mm length, 5 µm fully porous column. This reduction in column length results in cost savings by decreasing run time and solvent consumption.

Core-Shell & Fully Porous (cont'd)

What is the backpressure limitation for Kinetex core-shell particles?

If the column has an ID of 4.6 mm. then the max backpressure will be 600 bar, regardless of the particle-size. This has more to do with the column packing within a 2.6 mm ID column.

The 5 µm Kinetex particles should not exceed 600 bar regardless of column hardware dimensions.

Is there a difference in the backpressure created from core-shell particles and fully porous particles of the same size?

Generally, not much difference in backpressure based on particle-morphology. The backpressure is strictly limited to the particle-size and flow-rate (and mobile-phase viscosity).

The 2.1 mm ID columns can reach up to 1034 bar if the column is packed with 1.3, 1.7, or 2.6 µm Kinetex particles.

How does the backpressure of Kinetex 1.7 µm core-shell compare to 1.7 µm fully porous columns?

1.7 µm core-shell columns result in backpressures that are comparable to other sub-2 µm columns in the market. A UHPLC system is required when using a Kinetex 1.7 µm core-shell column.

How does the backpressure of Kinetex 2.6 µm core-shell compare to 3 and 5 µm fully porous columns?

Under the same running conditions and column dimension a 3.5x increase (vs. 5 µm fully porous particle) and a 1.3x increase (vs. 3 µm fully porous particle) in backpressure is typically observed. However, the increased performance provided by Kinetex 2.6 µm columns allows the use of shorter length columns to mitigate increases in backpressure. For an estimate of the backpressure you will experience when using a Kinetex 2.6 µm column on your current method visit **The Kinetex Calculator**.

How does the backpressure of Kinetex 2.6 µm core-shell compare to 1.7 µm fully porous columns?

Kinetex 1.7 µm core-shell columns result in backpressures that are comparable to other sub-2 µm columns in the market. Chromatographers can expect lower backpressure when using a Kinetex 2.6 µm vs. Kinetex 1.7 µm column.

How does the performance of Kinetex 2.6 µm core-shell columns compare to 3 µm and 5 µm fully porous columns?

2.6 µm particles provide roughly 3x the efficiency of 5 µm fully porous particles and 2x the efficiency of 3 µm fully porous particles without the need for specialized, high pressure instrumentation.

I have switched from a 5 µm fully porous column to a 2.6 µm Kinetex core-shell column but am seeing a loss of sensitivity, how can I overcome this?

When switching to a higher efficiency material such as Kinetex, you must take steps to ensure your detector scan rate has been adjusted appropriately to accommodate the narrower peak widths that the Kinetex column will generate. A detector scan rate of 1 Hz will generate 1 data point per second so a 20s wide peak is sufficient to ensure you do not see flattening of your peak at the apex. When you move to a more efficient media with narrower peaks, you must turn up the detector scan rate to accommodate the narrower peak width and prevent loss of the apex of the peak which can appear as a loss of peak efficiency.

I'm still unsure about whether or not Kinetex core-shell technology will realize its theoretical potential in practice. Do you have any third party references or case studies?

Yes. A number of peer reviewed journal articles have explored the theoretical and observed performance of Kinetex core-shell technology and compared it to other HPLC and UHPLC products on the market.

1. Gritti, Fabrice, Irene Leonardis, Jude Abia, and Georges Guiochon. "Physical Properties and Structure of Fine Core–shell Particles Used as Packing Materials for Chromatography." Journal of Chromatography A: 3819-843

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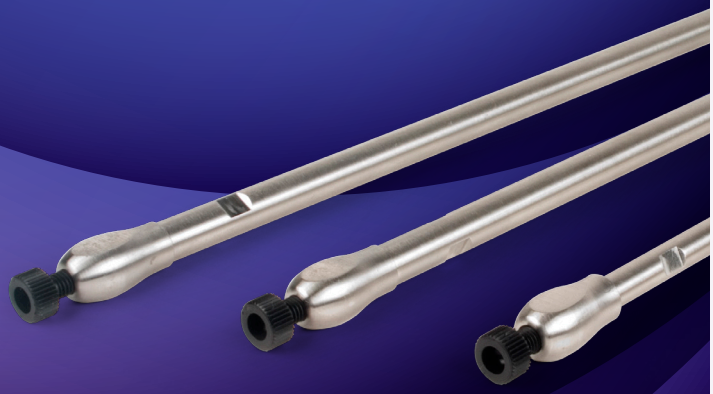

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SecurityGuard is patented by Phenomenex. U.S. Patent No. 6,162,362
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