

An Ounce of Prevention Keeping Your HPLC Column in Tip Top Shape

Jean Lane Application Engineer LC Columns and Consumables Technical Support February 24, 2022





Steps For Easy HPLC



- Purchase your HPLC column for your application
- Receive your HPLC column
- Remove column from the packaging
- Remove the column's end plugs
- Install the HPLC column
- Run it

It's that simple, correct?

Not so fast



An Ounce of Prevention Outline



To keep your column in tip top shape and to maximize column lifetime, there are a few things we should take into consideration...

1	Before the run starts
	 Why it is important to avoid solvent contamination Why and when to use inline filters and guard columns How tubing or bad fitting connections can cause problems
2	During operation
	 Installing your HPLC column How backpressure influences column lifetime How low and high pH can cause column failure
3	After the last run is finished
	How to flush LC columnsHow to properly store columns





Protect Your Column Before the Run Starts







Protect Your Column Before a Run



Why it is important to avoid solvent contamination

Example: Water from Lab Purification System

Purified water after discarding several liters



Purified water after one weekend



Contaminated solvents can lead to:

- Partially blocked frits and filters, causing
 - Increased column backpressure
 - Increased pump backpressure
 - Peak splitting and broadening
- Change in column selectivity and performance



Important to know

To prevent microbial growth in your aqueous mobile phase, prepare, filter, and degas mobile phases on a daily basis.

If the instrument is not used over a longer period of time, properly flush the instrument first with water to remove buffer residues, then with at least 10% IPA (or MeOH, ACN) in water.



Protect Your Column Before a Run







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Instrument as a Source of Particulates





InfinityLab Solvent Filtration Assembly PN 3150-0577

Instrument and solvents as a source of particulates

- Filter buffered LC solvents with Agilent Solvent Filtration equipment to remove precipitated/undissolved salts
- Use Agilent inline filters in the pump to remove pump seal wear

Filter membrane types

- Regenerated cellulose 47 mm, 0.45 µm
- Nylon
- PTFE

47 mm, 0.45 μm

 $47 \text{ mm}, \, 0.45 \, \mu\text{m}$

Consumable parts for assembly are sold separately

Solvent inlet filters





Glass filter, 20 µm pore size, p/n 5041-2168 Stainless steel filter, 10 µm pore size, p/n 01018-60025



Why Use an Inline Filter?





Accelerated lifetime test shows how inline filter removes particles

Pressure



InfinityLab Quick Change Inline Filter







Ultimate ease-of-use

- Finger-tight, tool-free replacement of filter disc
- Click and seal: A click alerts users when the filter is tight up to 1300 bar, assuring no risk of over- or under-tightening

Robustness for low operational cost

Robust filter housing enables **over 100 replacements** of filter discs without any damage

Plate counts over x100 installations of filter discs into one filter housing





InfinityLab Quick Change Inline Filter – Filter Discs

Infinity Lab

High efficiency, easy-to-use filter discs

- Various dimensions and porosities filter discs are available in 2.1 mm and 4.6 mm inner diameters with different pore sizes. The filter housing is compatible with all types of filter discs.
- **Touchless packaging to avoid potential contamination** with specially designed packaging, you're able to insert the filter disc into the filter housing without touching it, to avoid potential contamination.
- In situ replacement of filter disc no need to disconnect the inline filter from the system
- Smart alert to remind users when filter discs need replacing



Different dimensions and porosities of filter discs



Filter discs in touchless packaging



No-touch insertion of filter disc into filter housing







Protect Your Column Before a Run



How particle and matrix components can block your LC column



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Agilent application note: 5994-1947EN

Sample as a Source of Particulates



Sample as a source of particulates

- Filter sample with Agilent syringe filters to remove particulates
- Use Agilent inline filters and / or guard columns to protect from injector seal wear and sample compounds precipitating in gradient starting conditions



Agilent syringe filters







Agilent Quick Change inline filters



Sample Preparation Filtration

- Filtration for particulate removal
 - Captiva premium syringe filters
 - Captiva filter vials
 - Captiva filter plates and cartridges
- Filtration for lipid, proteins, and particulate removal
 - Captiva EMR–Lipid plates and cartridges









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Filtration

Captiva premium syringe filters

- Certified to be free of UV-detectable extractables on HPLC. PES and glass fiber are also certified for LC/MS.
- Color-coded boxes for easy identification
- Comprehensive portfolio to meet customer needs

Premium Syringe Filters							
Membrane	Diameter/Pore Size						
	4 mm		15 mm		25 mm	(28 mm)	
	0.2 µm	0.45 µm	0.2 µm	0.45 µm	0.2 µm	0.45 µm	
PTFE	•	•	•	•	•	•	
Nylon			•	•	•	•	
PES	•	•	•	•	•	*	
Regenerated cellulose	•	•	•	•	•	•	
Cellulose acetate					•	*	
Glass microfiber			•		•		
Depth filters: glass/PTFE			•	•	•	•	
Depth filters: glass/nylon			•	•	•	•	





Captiva syringe filters guide 5991-1230EN





The Value of Guard columns



Guards protect your column in many ways

What They Do

- Provide protection for the analytical column
 - Filter particulates
 - Unwanted chemical contamination
 - Protect against pressure spikes
- Increase total "column length"
 - Increase retention
- Should increase efficiency

What They Don't Do

- Replace good sample cleanup
- Replace column hygiene

It is not a "magic device"



Video: Fast Guards for HPLC and UHPLC | Agilent



InfinityLab Fast Guard





Agilent Guard Cartridge and holder

Guard Columns

Infinity Lab

Guards protect your column in many ways





*Unfiltered infant formula including proteins and other precipitated ingredients.





An Ounce of Prevention

Considerations for system tubing and column connections





System Tubing Reducing dead volume







- Peak shape is poor
- Resolution between peaks is poor

- Use proper fittings
- Keep tubing connections short
- Inner diameter for tubing should be as narrow as possible



Capillary Tubing Volume





Tubing Length	10 mm	50 mm	100 mm	150 mm
Tubing id	Volume	Volume	Volume	Volume
0.17 mm (green)	0.227 μL	1.1 μL	2.27 µL	3.3 µL
0.12 mm (red)	0.113 µL	0.55 µL	1.13 µL	1.65 μL



Optimizing Connecting Tubing Volume for UHPLC Columns









System Fittings Potential fittings issues





- Leak
- Peak shape problem

No dead volume

Connection problems can lead to:

Poor chromatography

- Broad or tailing peaks
- Loss of resolution

Added maintenance costs

- Leaks, added troubleshooting
- Overtightening
- Column damage



Poor Connections Fitting Mismatch











Overtightened fittings







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Have a Poor Fitting Connection? Peak shape issue





InfinityLab Quick Connect and Quick Turn Fittings





	Quick Connect Fitting	Quick Turn Fitting
Connects to	Columns (or inline filters)	Column, various receiving ports with 10-32 port geometry
Maximum pressure	1300 bar (finger-tight, by turning the lever)	300 to 400 bar (finger-tight, user-dependent) 1300 bar (with mounting tool, 5043-0915) Bio-inet mounting tool, 5043-0915
Features	 Spring-loaded function for dead volume free connections (special capillaries) Replaceable ferrule and capillary Capillaries in various lengths and diameters are available 	 Spring-loaded function for dead volume free connections Replaceable ferrule and capillary Capillaries in various lengths and diameters are available
Wetted material	PEEK (ferrule)	PEEK (ferrule)





InfinityLab Quick Connect Fittings Component overview

It is important that Quick Connect fittings are only used with capillaries specially designed for them, to ensure proper function.



All capillaries come without any fittings, except the ones with a single preswaged M4 fitting for the opposite end.





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During Operation

Column installation, roles of mobile phase, and considerations for back pressure and pH





An Ounce of Prevention Refer to the column user guide





COLUMN USER GUIDE for Agilent Reversed-Phase Columns

Agilent 反相色谱柱用户指南

Guide d'utilisation des colonnes pour les colonnes à phase inverse Agilent

Säulenbenutzerhandbuch für Agilent Umkehrphasensäulen

Manuale utenti per colonne a fase inversa Agilent

Guía de usuario de columna para columnas de fase reversa Agilent

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ZORBAX reversed-phase / 反相 / à phase reversa / Umkehrphase / Fase inversa / Fase inversa / 逆相

HPLC phases / 相 / Phases HPLC / Phasen / Fasi / Fases /カラム

Poroshell 120/300 / Pursuit / Polaris / HC-C18(2) / TC-C18(2)

User guide provides information for:

- Shipping solvent
- Technical specifications
- Column conditions
- Mobile phases guidelines
- Recommended starting gradients
- Column care
- Tips for getting the best chromatography
- Troubleshooting
- Column cleaning and storage





820000-999-LCUserGuide-Zorbax and InfinityLab Poroshell 120 Reverse Phase columns.pdf (agilent.com)

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Take Care Installing Your Column



- Set maximum back pressure for your system which will be appropriate for your column
- Check the solvent that the column was shipped in
- Know what mobile phase is currently running in your LC system
- Care should be taken not to pass any mobile phase through the column that may cause a precipitate to form
- If mobile phase additives are used (such as buffers or ion-pair reagents) it is advisable to do an intermediate flush with a mobile phase of the correct composition, but without these additions
- Flush the LC system with mobile phase through to the column inlet connection
- Note the correct flow direction on column when connecting it to the LC system.
- It is advised to introduce the mobile phase to the column at a reduced flow rate while monitoring back pressure
- Gradually increase the flow rate of your column to the working flow rate and allow for column equilibration



Before We Start Our Analysis



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Every new column should be "QC" tested on your instrument



Performance verification based on Agilent checkout

- Run Agilent checkout before use
 - Record difference between your instrument and performance report (use as base value)
- Perform again if column seems to lose performance
 - Compare with results from first run

Performance verification based on in-house checkout

- Run in house checkout before use
 - Record key specifications such as tailing factor, plates, backpressure
- Perform again if column seems to lose performance
 - Compare with results from first run

How Backpressure Can Influence Column Lifetime



- Aqilent

Two kinds of pressure changes that can lead to column failure



During every injection, the sample loop gets switched into the flow patch. The pressure of the sample and their compressibility is often different, which leads to a pressure drop. The more sample injected and the higher the method backpressure, the higher the pressure drop. Bigger sample loops lead to higher pressure drops.



With changes in the mobile phase composition but constant flow rate, system pressure changes along the gradient. This leads to constantly changing pressure at the column inlet and along the column, which negatively affects column lifetime.



Never use the entire pressure range. Allow 10-15% of maximum instrument and column pressure as a buffer to:

- Improve column lifetime as pressure fluctuations can be better absorbed
- Keep instrument runtime up

Never develop your method on a single column. Use method validation kits with columns from three batches to get a feeling of the backpressure variation between batches – especially for older products.



What affects lifetime of silica-based columns under high pH?

- Previous studies have shown that bonded-phase packing degradation at pH 9-10 mainly is due to silica support dissolution, not primarily from the hydrolysis of covalently attached siloxane bonds.
- Column lifetime is a function of concentration of sodium>potassium >ammonium cations.
- Bonded-phase packing degradation is higher at pH 7-10 when concentrations of certain buffers are higher, especially phosphate.
- Column degradation at high pH is strongly influenced by temperature.

H. A. Claessens, M. A. van Straten, and J. J.Kirkland, J. Chromatogr. (A), 728 (1996) 259





The high pH failure mode of an LC column



Retention Time

LC method parameters
InfinityLab Poroshell 120 EC-C18, 2.1 x 50 mm, 2.7 μm
Sample: Agilent Basic Pesticide Mixture Solvents:

A: Water
B: Acetonitrile
C: 500 mL of 200 mM (NH₄)₂CO₃ + 50 mL of 30% NH₄OH, pH 10.2

Gradient: 15-60% B in 7 min, 20% C Injection volume: 0.6 μL Column compartment temperature: 60 °C Flow rate: 0.4 mL/min







2.0

2.5

3.0

1.5

3.5

4.5





The right LC column for high pH separations

InfinityLab Poroshell 120 column specifications

InfinityLab Poroshell Family		Pore Size	Temp. Limits	pH Range	Endcapped	Carbon Load	Surface Area
Post all around	EC-C18	120 Å	60 °C	2.0-8.0	Double	10%	130 m2/g
Best all around	EC-C8	120 Å	60 °C	2.0-8.0	Double	5%	130 m2/g
Past for low pH mobile phases	SB-C18	120 Å	90 °C	1.0-8.0	No	9%	130 m2/g
Best for low-pri mobile phases	SB-C8	120 Å	80 °C	1.0-8.0	No	5.5%	130 m2/g
Past for high pH mobile phases	HPH-C18	100 Å	60 °C	3.0-11.0	Double	Proprietary	95 m2/g
Best for high-pH mobile phases	HPH-C8	100 Å	60 °C	3.0-11.0	Double	Proprietary	95 m2/g
	HILIC	120 Å	60 °C	0.0-8.0	N/A	N/A	130 m2/g
Best for polar compounds (HILIC)	HILIC-Z	120 Å	80 °C	3.0-11.0	Proprietary	Proprietary	130 m2/g
	HILIC-OH5	120 Å	45 °C	1.0-7.0	Double	Proprietary	130 m2/g
	Bonus-RP	120 Å	60 °C	2.0-9.0	Triple	9.5%	130 m2/g
	PFP	120 Å	60 °C	2.0-8.0	Double	5.1%	130 m2/g
Best for alternative selectivity	Phenyl-Hexyl	120 Å	60 °C	2.0-8.0	Double	9%	130 m2/g
	SB-Aq	120 Å	80 °C	1.0-8.0	No	Proprietary	130 m2/g
	EC-CN	120 Å	60 °C	2.0-8.0	Double	3.5%	130 m2/g
	Chiral-T	120 Å	45 °C	2.5-7.0	Proprietary	Proprietary	130 m2/g
Reat for chiral concrations	Chiral-V	120 Å	45 °C	2.5-7.0	Proprietary	Proprietary	130 m2/g
best for child separations	Chiral-CD	120 Å	45 °C	3.0-7.0	Proprietary	Proprietary	130 m2/g
	Chiral-CF	120 Å	45 °C	3.0-7.0	Proprietary	Proprietary	130 m2/g





Hybridized superficially porous silica particle (HPH-C18). Synthesized by a proprietary process.

From Agilent HPLC conference poster, 2014.



Infinity Lab

Low pH (1 to 3) – Bonded phase loss by acid catalyzed hydrolysis

Conventional

StableBond





* Hydrolytically sensitive siloxane bond





The right LC column for low pH separations

InfinityLab Poroshell 120 column specifications

	InfinityLab Poroshell Family		Pore Size	Temp. Limits	pH Range	Endcapped	Carbon Load	Surface Area
	Deat all accord	EC-C18	120 Å	60 °C	2.0-8.0	Double	10%	130 m2/g
	Best all around	EC-C8	120 Å	60 °C	2.0-8.0	Double	5%	130 m2/g
	Best for low-pH mobile phases	SB-C18	120 Å	90 °C	1.0-8.0	No	9%	130 m2/g
		SB-C8	120 Å	80 °C	1.0-8.0	No	5.5%	130 m2/g
	Deat for high all makile above	HPH-C18	100 Ă	60 °C	3.0-11.0	Double	Proprietary	95 m2/g
	Best for high-pH mobile phases	HPH-C8	100 Å	60 °C	3.0-11.0	Double	Proprietary	95 m2/g
	Best for polar compounds (HILIC)	HILIC	120 Å	60 °C	0.0-8.0	N/A	N/A	130 m2/g
		HILIC-Z	120 Å	80 °C	3.0-11.0	Proprietary	Proprietary	130 m2/g
		HILIC-OH5	120 Å	45 °C	1.0-7.0	Double	Proprietary	130 m2/g
	Best for alternative selectivity	Bonus-RP	120 Å	60 °C	2.0-9.0	Triple	9.5%	130 m2/g
		PFP	120 Å	60 °C	2.0-8.0	Double	5.1%	130 m2/g
		Phenyl-Hexyl	120 Å	60 °C	2.0-8.0	Double	9%	130 m2/g
		SB-Aq	120 Å	80 °C	1.0-8.0	No	Proprietary	130 m2/g
		EC-CN	120 Å	60 °C	2.0-8.0	Double	3.5%	130 m2/g
		Chiral-T	120 Å	45 °C	2.5-7.0	Proprietary	Proprietary	130 m2/g
	Deat for object concretions	Chiral-V	120 Å	45 °C	2.5-7.0	Proprietary	Proprietary	130 m2/g
	Best for chiral separations	Chiral-CD	120 Å	45 °C	3.0-7.0	Proprietary	Proprietary	130 m2/g
		Chiral-CF	120 Å	45 °C	3.0-7.0	Proprietary	Proprietary	130 m2/g

Specifications represent typical values only





Low stability lifetime test on InfinityLab Poroshell 120 SB-C18



	Efficiency change (%) after 20K column volume under pH 1
Plates	-0.6%
Retention Time	-3.9%
Tailing Factor	<u> </u>

+0.5%

LC method parameters

InfinityLab Poroshell 120 SB-C18, 2.1 x 50 mm, 2.7 µm

Instrument: 1260 Infinity II Sample: Uracil and toluene Solvent: 50/50 methanol / 2%TFA in water, pH=1 Injection volume: 1 µL Column compartment temperature: 85 °C Flow rate: 0.3 mL/min Continuous injection every 60 min



Poroshell 120 Family Offers Chemistries with Unique Selectivity Infinity

InfinityLab Poroshell 120 offers a broad portfolio to suit your needs

	For Nonpo				
Best All Around	Best for Low pH Mobile Phases	Best for High pH Mobile Phases	Best for Alternative Selectivity	Best for More Polar Analytes	Chiral
EC-C18 1.9 μm, 2.7 μm, 4 μm	SB-C18 1.9 μm, 2.7 μm, 4 μm	HPH-C18 1.9 μm, 2.7 μm, 4 μm	Bonus-RP 2.7 μm	SB-Aq 1.9 μm, 2.7 μm, 4 μm	Chiral-V 2.7 μm
EC-C8 1.9 μm, 2.7 μm, 4 μm	SB-C8 2.7 μm	HPH-C8 2.7 μm, 4 μm	PFP 1.9 μm, 2.7 μm, 4 μm	EC-CN 2.7 μm	Chiral-T 2.7 μm
Phenyl-Hexyl 1.9 μm, 2.7 μm, 4 μm		CS- 2.7	C18 μm	HILIC 1.9μm, 2.7 μm, 4 μm	Chiral-CD 2.7 μm
	For RP start here			HILIC-Z 1.9 μm, 2.7 μm, 4 μm	Chiral-CF 2.7 μm
				HILIC-OH5 2.7 μm	





ab



After the Last Run is Finished

How to properly flush and store LC columns







Calculating Column Volume





$$V = \pi r^2 h$$

- 1. Volume of a cylinder
- 2. You'll also need an estimation of the void volume of the column; 60% is considered reasonable

Example calculation – column size 4.6 x 150 mm

1. cm^3 ($\pi \times 0.23$ cm $\times 0.23$ cm $\times 15$ cm), which is 2.49 cm³ or 2.49 mL (remember 1 cm³ = 1 mL). 2. 2.49 x 0.6 = 1.5 mL

Volume for this column - 1.5 mL

You can also determine this for your column on your system by running an unretained sample.





How to Flush Your LC Columns



A stepwise protocol can help to remove blockages and contaminations

Tip 1: Flush with stronger solvents than your mobile phase

Tip 2: Use at least 10 column volume of each solvent for analytical columns

Tip 3: Make sure the detector is taken out of the flow path

Tip 4: To remove blockages or contaminations, you can try to backflush your

column (if supported – see your column user guide)





How to Flush Your LC Columns



A stepwise cleaning protocol can help to remove blockages or contaminations

Reversed-phase solvent choices in order of increasing strength

- 1. Mobile phase without buffer salts (water/organic)
- 2. 100% organic (MeOH or ACN)
- 3. Is pressure back in the normal range?
- If not, discard column or consider more drastic conditions:
 75% acetonitrile:25% isopropanol, then
- 5. 100% Isopropanol
- 6. 100% Methylene chloride*
- 7. 100% Hexane*

*Important to know

- When using either hexane or methylene chloride, the column must be flushed with isopropanol before returning to your reversed-phase mobile phase.
 Might also compromise instrument
- pump seal lifetime.



How to Properly Store Your LC Columns



A protocol for storing LC columns



COLUMN USER GUIDE for Agilent Reversed-Phase Columns

Agilent 反相色谱柱用户指南

Guide d'utilisation des colonnes pour les colonnes à phase inverse Agilent

Säulenbenutzerhandbuch für Agilent Umkehrphasensäulen

Manuale utenti per colonne a fase inversa Agilent

Guía de usuario de columna para columnas de fase reversa Agilent

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HPLC phases / 相 / Phases HPLC / Phasen / Fasi / Fases /カラム

Poroshell 120/300 / Pursuit / Polaris / HC-C18(2) / TC-C18(2)

Always follow the recommendations in the LC Column user guide

User guides for Agilent columns are available on <u>Agilent.com</u> or by entering "Agilent LC Column user guide" into your search engine

- Long-term storage of RP LC columns should be in a pure organic solvent. If the column has previously been used with a buffered mobile phase, the buffer should first be removed by purging the column with 20 to 30 column volumes of a 50:50 mixture of methanol or acetonitrile and water, followed by 20 to 30 column volumes of the pure solvent.
- 2. Before storing, end-fittings should be tightly capped with end-plugs to prevent packing from drying out.
- 3. To protect equipment, is it desirable to remove salts from the instrument and column by purging the column with the same mobile phase without the buffer.

Review of Good Column Practices



✓ Filter buffers

- ✓ Investigate effects of sample solvent on solubility and separation
- ✓ Filter or pretreat samples that contain strongly retained components of no interest.
- ✓ Use fresh aqueous mobile phase solutions
- ✓ Use inline filters
- ✓ Use appropriate guard column
- ✓ Use proper size id tubing and correct fittings for LC and column connections
- ✓ Be aware of column packing specifications and limits. Example:
 - Solvent and chemical compatibility
 - Temperature
 - рН
- ✓ Flush column periodically with strong solvent
- ✓ To store column, purge buffers and leave in appropriate storage solvent (for example, ACN)
- ✓ Avoid physically mishandling columns: banging, dropping, or overtightening fittings



LC Columns and Supplies Resources

- LC column user guides: <u>LC Column user guides | Agilent</u>
- BioLC column user guides: <u>Bio LC Column user guides | Agilent</u>
- InfinityLab supplies catalog: InfinityLab LC Supplies (agilent.com)
- LC Handbook: <u>LC-Handbook-Complete-2.pdf (Agilent.com)</u>
- LC troubleshooting poster: LC Troubleshooting Guide (Agilent.com)
- Agilent Community: <u>Agilent Community</u>
- Consumables Community: <u>Agilent Collection of Columns, Supplies, and Standards Resources -</u> <u>Consumables - Agilent Community</u>
- App finder: <u>Application Finder | Agilent</u>
- Agilent University: <u>Agilent University</u>
- YouTube: <u>Agilent Channel</u>
- Your local product specialists
- Agilent Peak Tales podcasts: peaktales.libsyn.com
- Webinars, upcoming and recorded: Webinars | Agilent







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Contact Agilent Chemistries and Supplies Technical Support





Available in the U.S. and Canada, 8-5 all time zones

1-800-227-9770 option 3, option 3:
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Option 3 for sample preparation, filtration, and QuEChERS
Option 4 for spectroscopy supplies
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Thank you for attending

Any questions?



