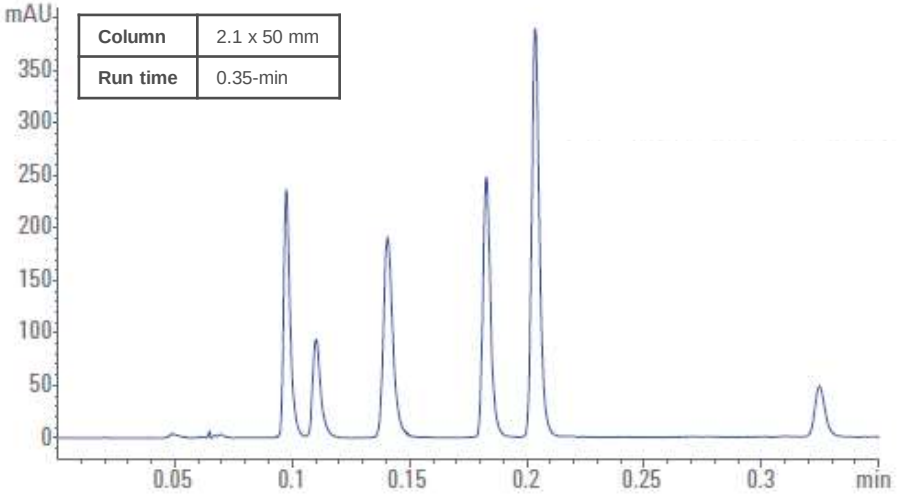
Am I getting the very best value from my UHPLC analyses?

Stephen Luke

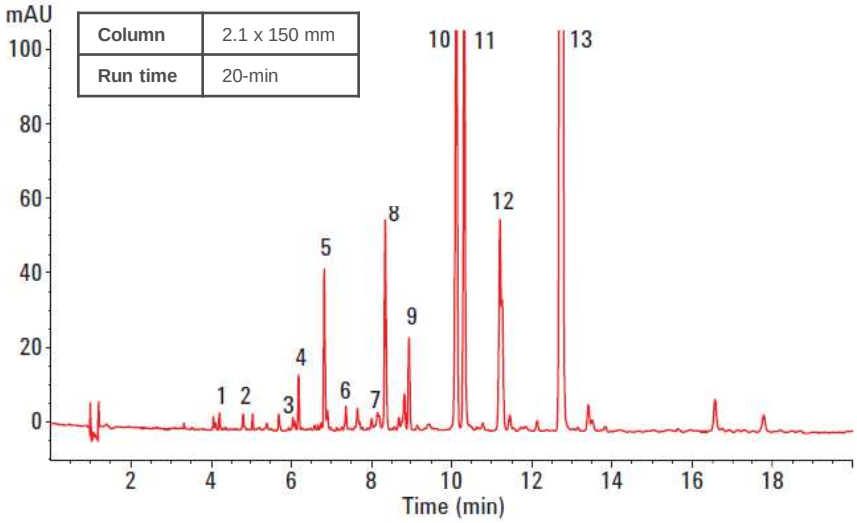
LC Columns Product Manager

Primary reasons for UHPLC use

Very fast



Very high resolution



Requirements for successful UHPLC

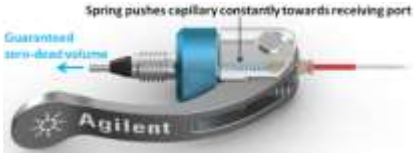
- The key requirement for UHPLC is very high efficiency separations
- This has consequences

Peaks are very narrow and have a low volume

- Fast enough detector sampling rate
- Very low instrument dispersion volume

Small particles are more efficient but generate high pressures

- High pressure rated columns and instruments



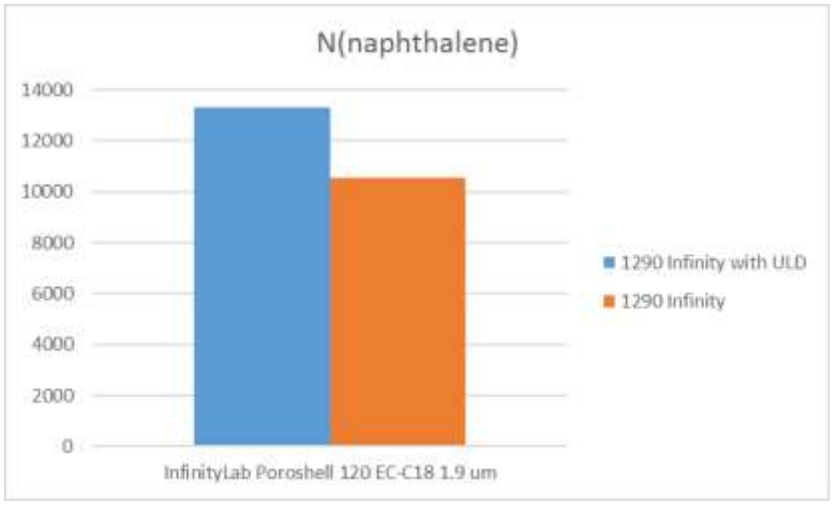
Minimize instrument dispersion volume

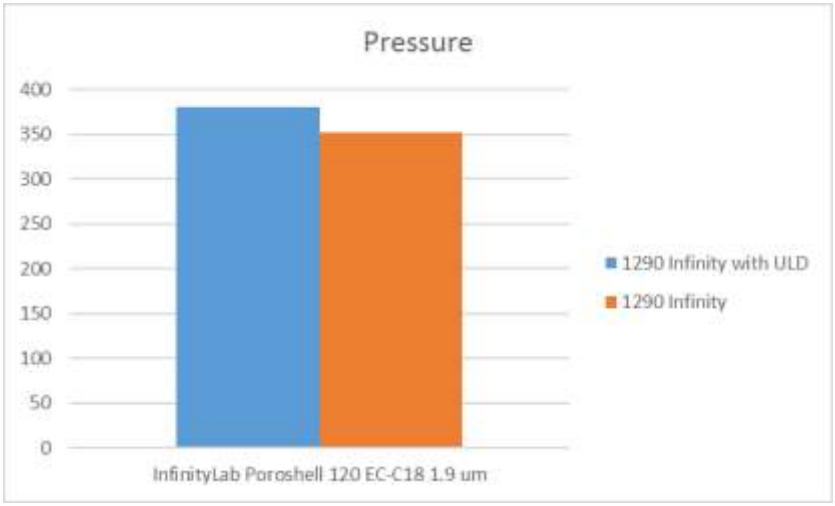
Agilent 1290 Infinity II Ultra Low Dispersion Kit

Part no	Description
G4267-87020	High Pressure Seat Assembly 0.075 mm (PEEK)
5500-1207	Capillary ST 0.075 mm x 500 mm - Multisampler to Heat exchanger
G7116-60021	Quick Connect Heat exchanger Ultra Low Dispersion
5067-6602	Quick Connect / Quick Turn Assembly ST 0.075 mm x 105 mm - Heat exchanger to column
5500-1208	Capillary ST 0.075 mm x 250 mm - Column outlet to Flow Cell

Note: The small diameter capillaries in this kit will significantly increase the backpressure of the system at high flow rates - for example +165 bar with water at 1 mL/min flow rate

Impact of ultra-low dispersion



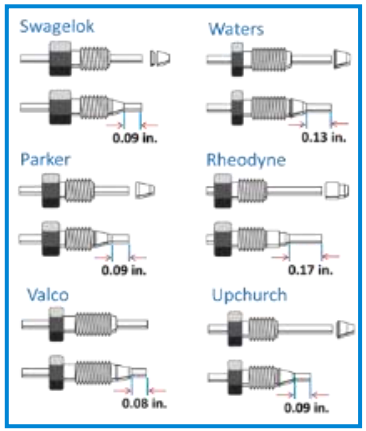


In this example the use of the ultra-low dispersion kit increased efficiency by 26%, with an 8% increase in pressure

2.1 x 50 mm columns, 40% 20 mM Sodium Phosphate pH 7, 55 or 60% Acetonitrile, 0.5 mL/min, 0.5 uL, 25C, 254 nm, 80 Hz

Minimize instrument dispersion - connections
Quick Connect and Quick Turn Fittings

Most commonly used fittings in UHPLC are non-adjustable 2-piece or 3-piece metallic fittings. Since different manufacturers of column hardware have different design in column end fittings, as shown in Figure 1, a new set of tubing and fittings needs to be installed for every brand of column to guarantee that the stem length, namely the length between the bottom of the ferrule and the end of tubing, fits the column end fitting.



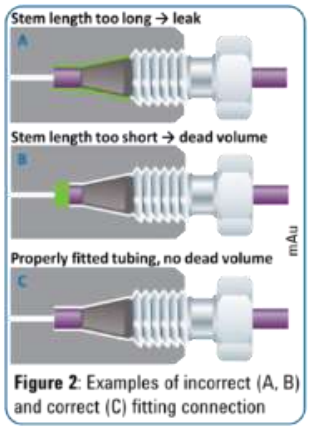


Figure 2: Examples of incorrect (A, B) and correct (C) fitting connection

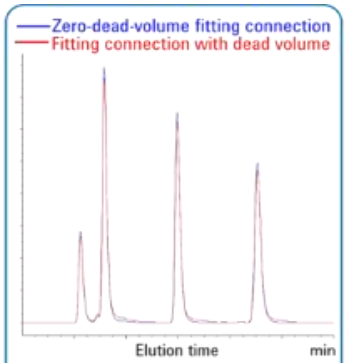
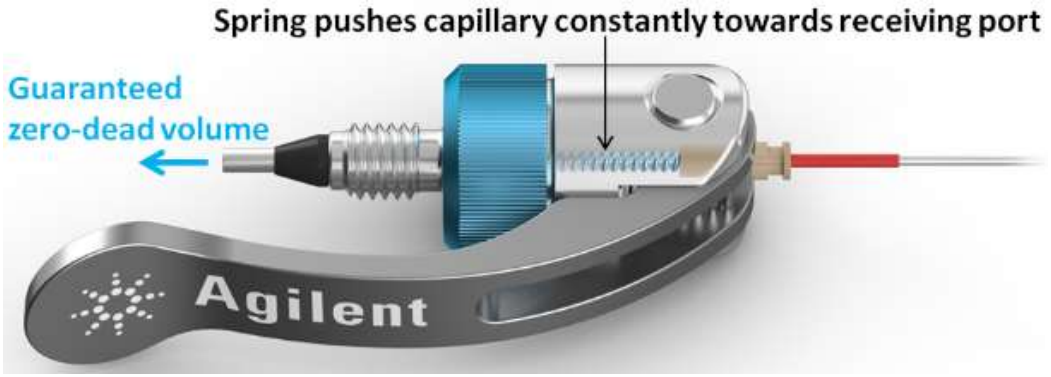


Figure 3: Comparison of chromatograms with correct and incorrect fitting

Importance of the Spring Loaded Feature

The spring-loaded design constantly pushes the tubing against the receiving port, delivering a reproducible connection with no dead volume for consistent chromatographic performance

Stem length is adjustable through the spring, which makes the fitting compatible with all types of LC columns.



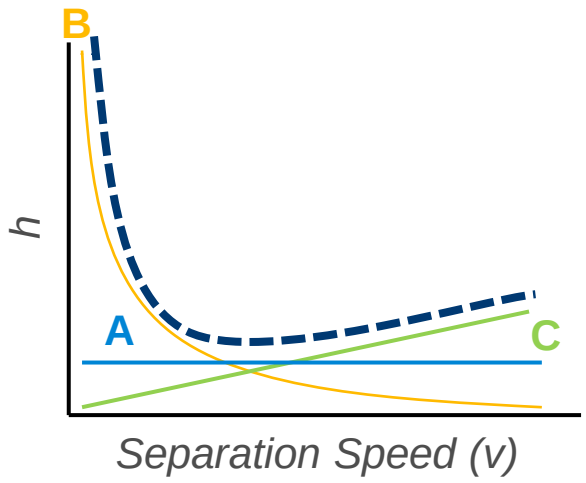
SPP = superficially porous particle

High efficiency separations

All 3 van Deemter terms are reduced with SPP

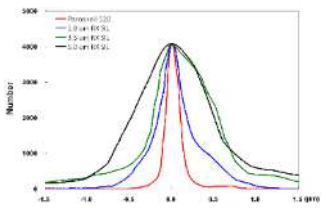
van Deemter equation:

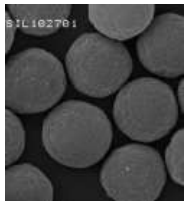
$$h = A + B/\nu + C \cdot \nu$$

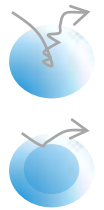


Lower *h* = higher efficiency!

- **A term – eddy diffusion**
  - **Particle size & packing quality**
  - **Narrow particle size distribution**
- **B term – longitudinal diffusion**
  - **Less mobile phase in the column**
  - **Reduced diffusion**
- **C term – mass transfer**
  - **Shorter diffusion paths**
  - **More effect on larger molecules**



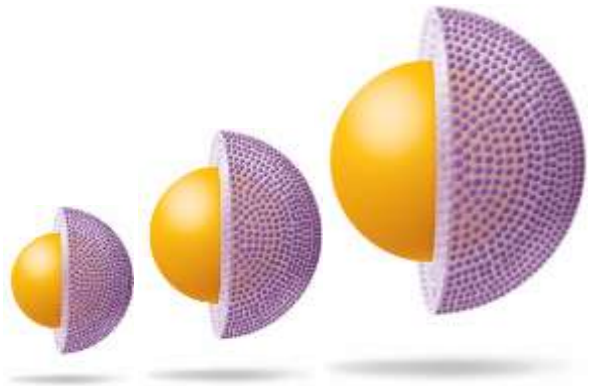


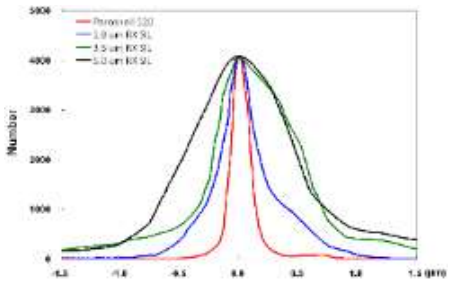


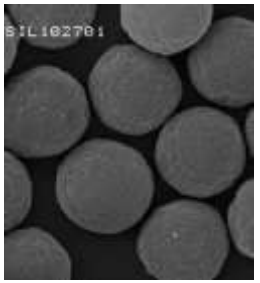
Higher efficiencies using SPP

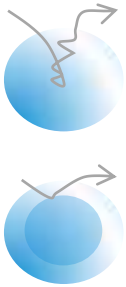
Additional efficiency can be generated through the use of superficially porous particles (SPP) rather than a totally porous particle (TPP)

SPP particle	For	Maximum pressure	Typical pressure	Efficiency
1.9 µm	Highest UHPLC performance	1300 bar	Similar to sub-2 µm totally porous	~120% of sub-2 µm totally porous
2.7 µm	UHPLC performance at lower pressures	600 bar	50% of sub-2 µm totally porous	~90% of sub-2 µm totally porous
4 µm	Improved HPLC performance	600 bar	Typically < 200 bar	~200% of 5 µm totally porous

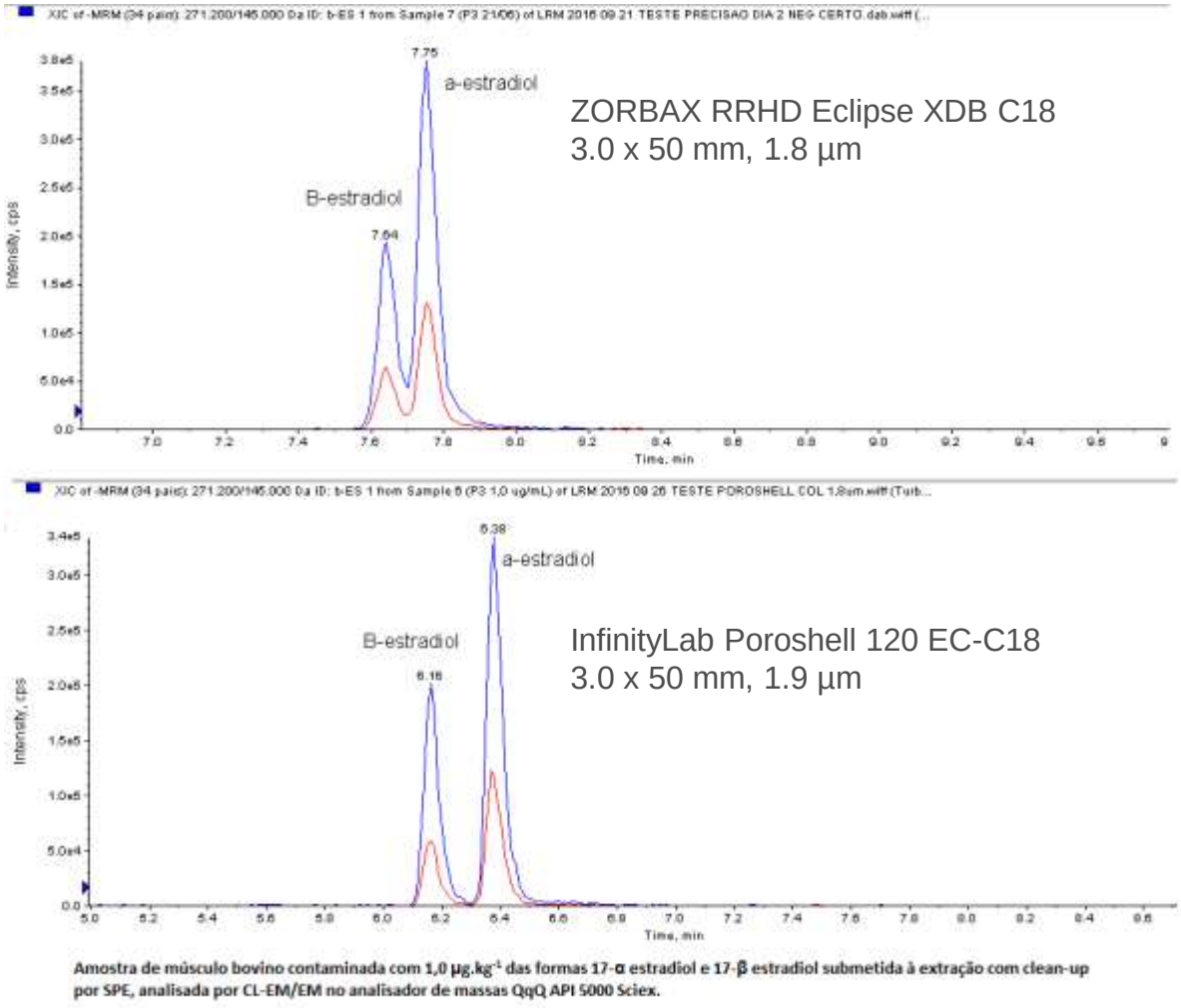








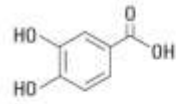
Higher efficiencies using SPP
Seed column feedback



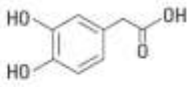
Amostra de músculo bovino contaminada com 1,0 µg.kg^{-1} das formas 17-α estradiol e 17-β estradiol submetida à extração com clean-up por SPE, analisada por CL-EM/EM no analisador de massas QqQ API 5000 Sciex.

"…the efficiency of the new Poroshell column was superior to Zorbax once achieved a good resolution in the separation of isomers of estradiol, essential for the validation of a method for monitoring such analytes. Thus, we will start to use the new Poroshell column in the ongoing validations in anabolic"
- Residue Laboratory Veterinary Medication LANAGRO / MG

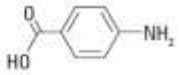
Fast LC
Aromatic acids



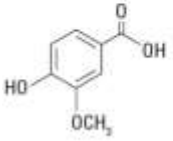
Protocatechuic acid



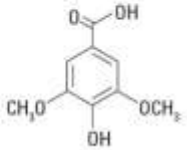
3,4-Dihydroxyphenylacetic acid (DOPAC)



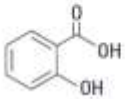
4-aminobenzoic acid (PABA)



Vanillic acid



Syringic acid



Salicylic acid

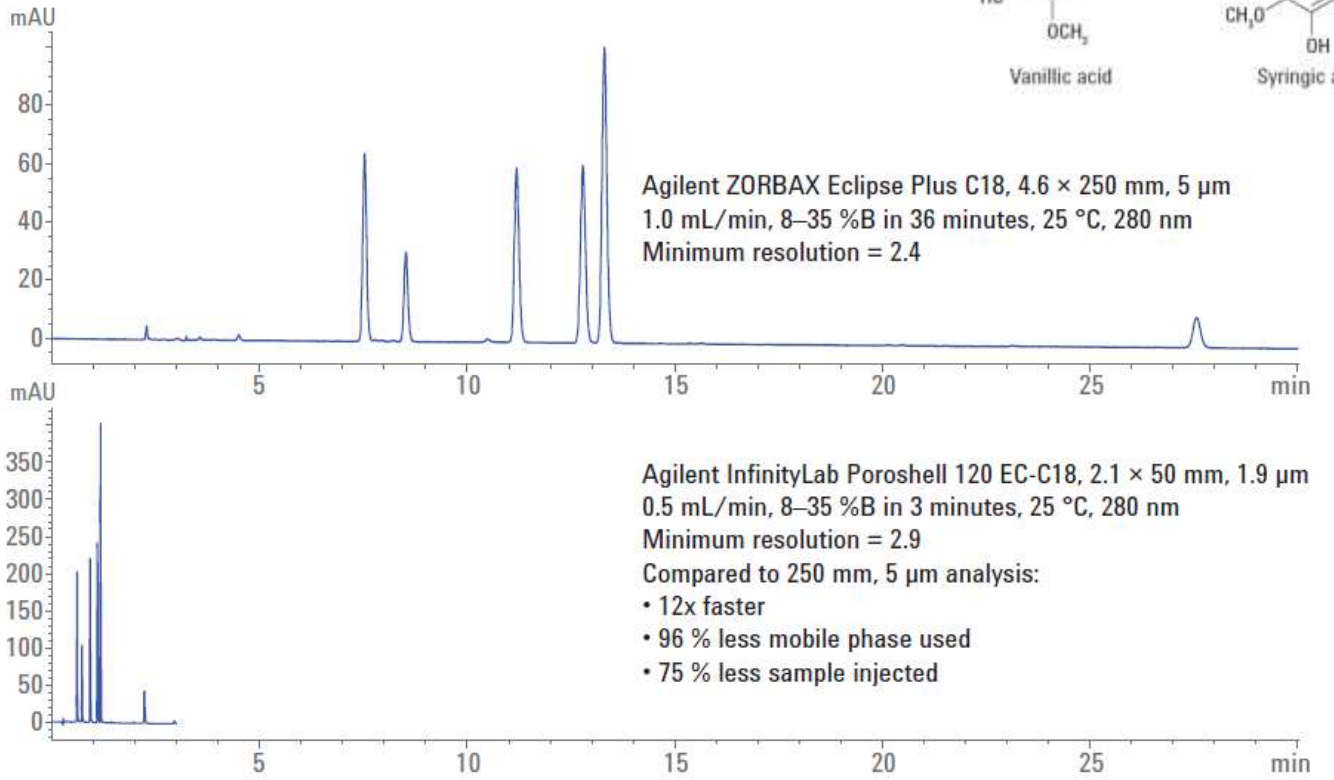


Figure 2. A 250 mm, 5 µm Agilent ZORBAX analysis of aromatic acids is improved by transferring to a high-performance 50 mm, 1.9 µm Agilent InfinityLab Poroshell column; minimum resolution is improved, while saving significant time, sample, solvent, and money.

5991-7518EN

Advantages of fast LC
Save time and reduce solvent use

Table 3. Comparison of aromatic acid analyses with different LC columns.

Column	Pressure (bar)	Minimum resolution	Conditional peak capacity (nC)	Run time (min)		Mobile phase consumption (mL)	
Agilent ZORBAX Eclipse Plus C18, 4.6 × 250 mm, 5 µm	193	2.4	88	36	Original 5 µm analysis	36	Original 5 µm analysis
Agilent Poroshell 120 EC-C18, 2.1 × 50 mm, 1.9 µm	472	2.9	58	3.0	12x Faster than original 5 µm analysis	1.5	Uses 96 % less mobile phase than original 5 µm analysis
Agilent Poroshell 120 EC-C18, 2.1 × 50 mm, 2.7 µm	281	2.1	52				
Agilent Poroshell 120 EC-C18, 2.1 × 50 mm, 4 µm	141	1.8	43				
Agilent Poroshell 120 EC-C18, 2.1 × 50 mm, 1.9 µm (ultrafast)	1150	2.3	37	0.35	103x Faster than original 5 µm analysis	0.77	Uses 98 % less mobile phase than original 5 µm analysis

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Ultra-fast LC
Aromatic acids

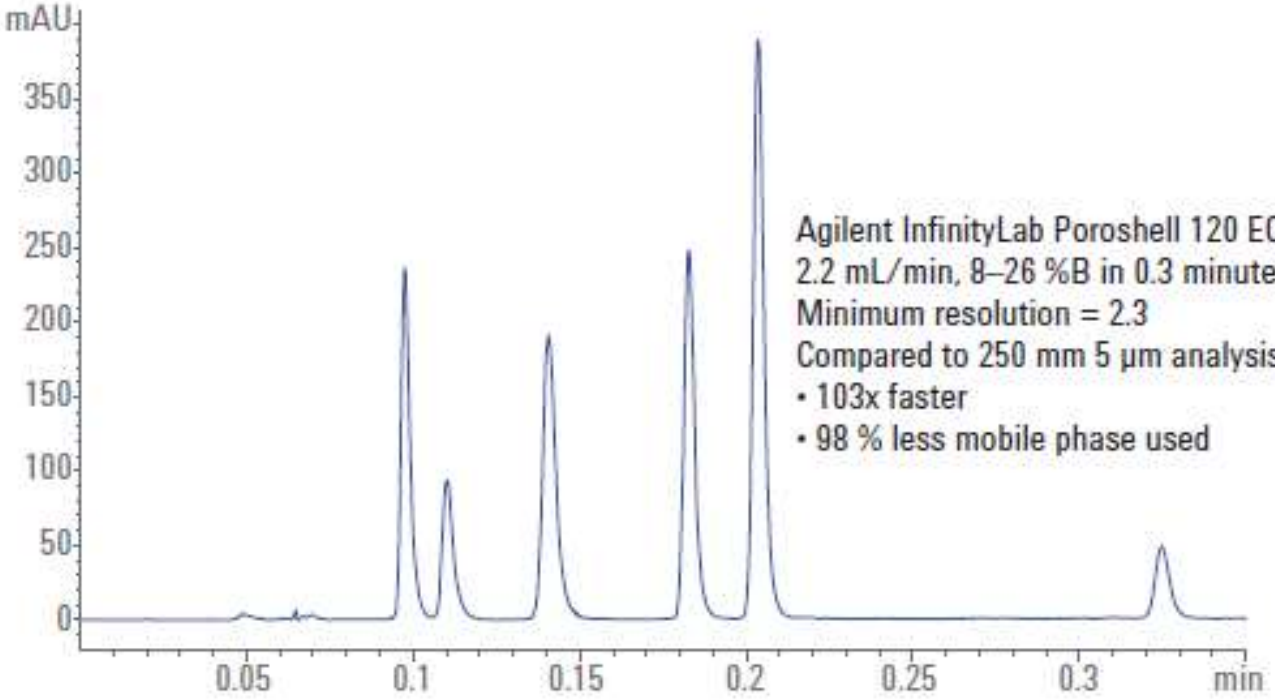


Figure 4. Additional time, solvent, and money can be saved by operating the highly performing 50 mm, 1.9 µm Agilent InfinityLab Poroshell column near its pressure limit without compromising method performance.

5991-7518EN

High resolution LC

Tanshinones in Danshen (Salvia miltiorrhiza)

$$n_c = (t_{R,n} - t_{R,1})/w$$

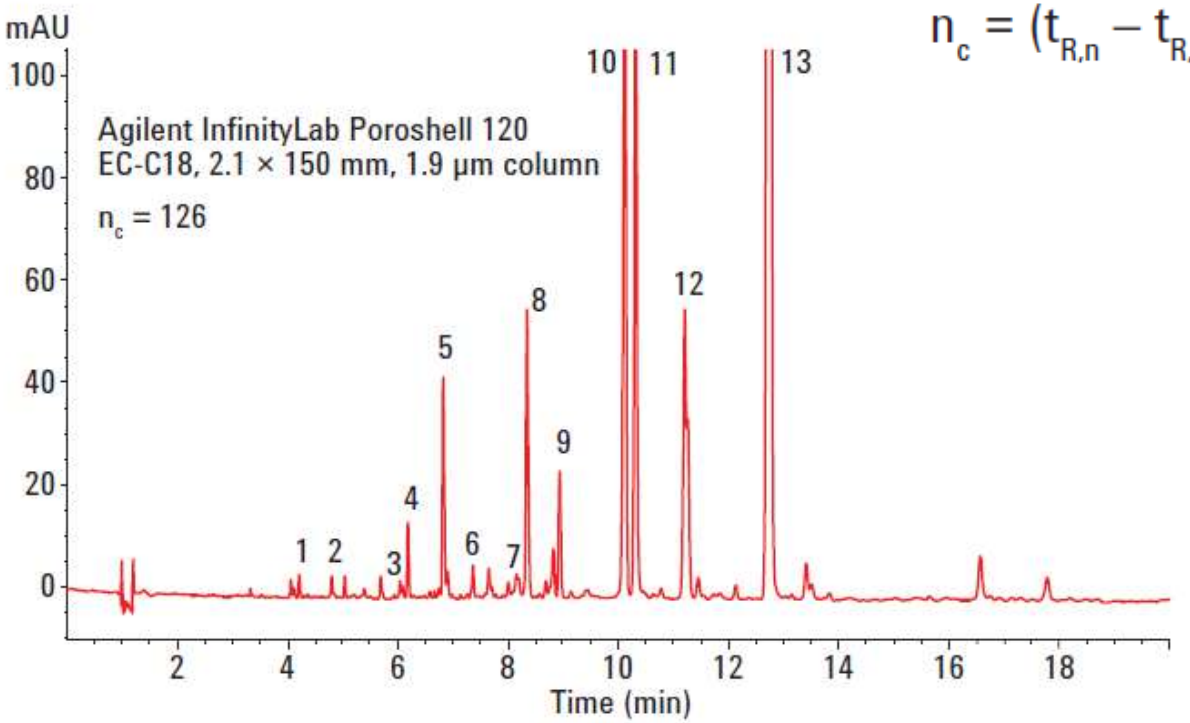


Figure 3. Tanshinones fingerprint profiling on an Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 150 mm, 1.9 µm column. Peaks 10 (cryptotanshinone) and 13 (tanshinone IIA) were identified using reference standards.

High efficiency column increases the number of peaks that can be resolved (n_c) and Improves the accuracy of fingerprinting

5991-7559EN

The advantage of longer columns

Total phenolic acids in Danshen (Salvia miltiorrhiza)

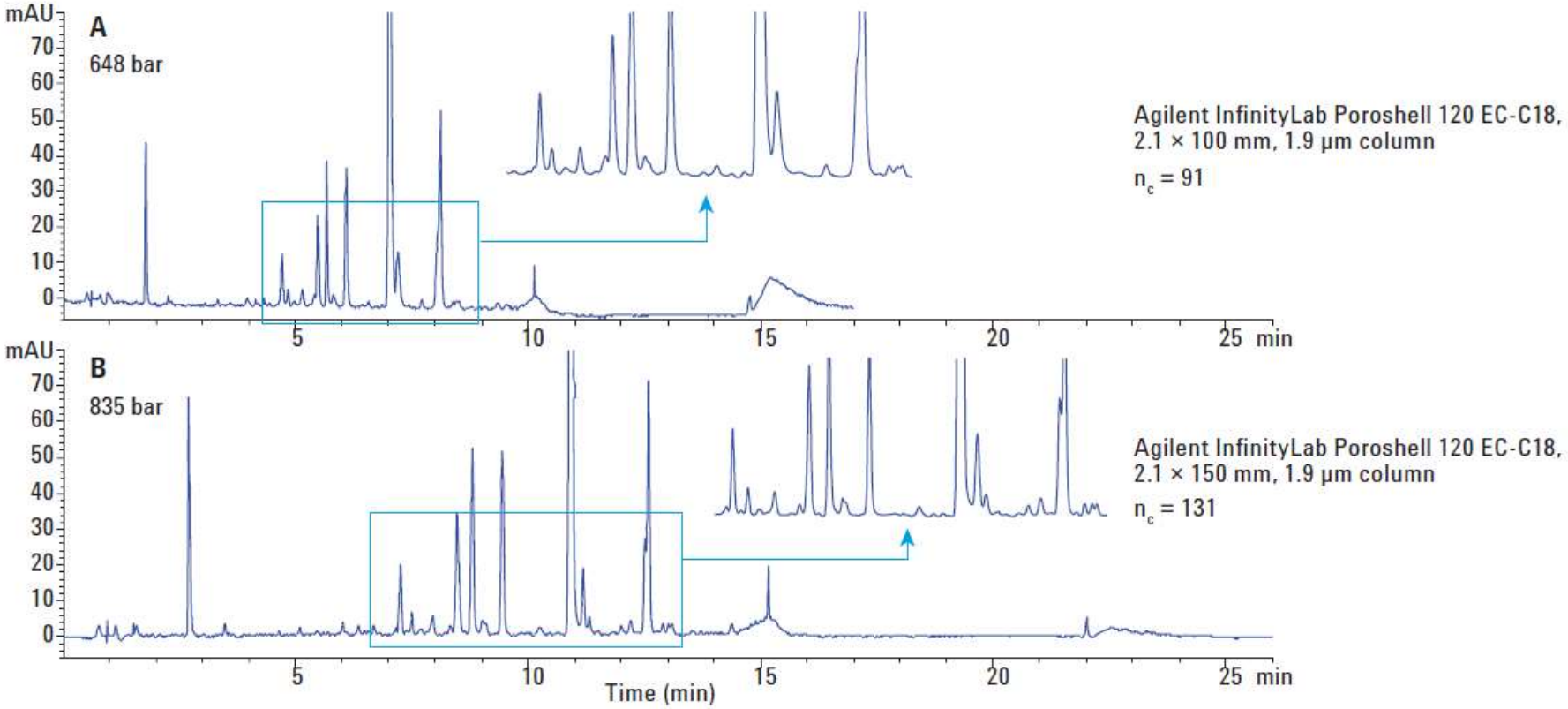
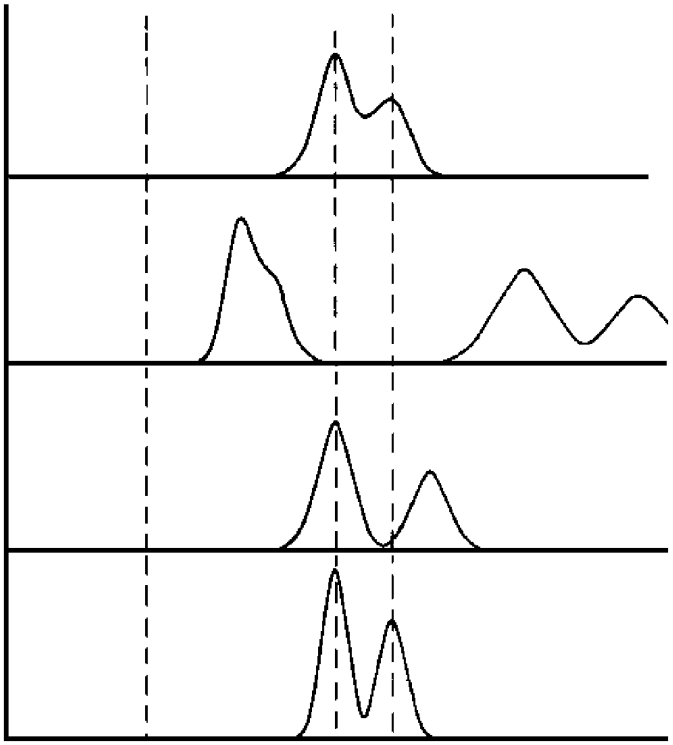


Figure 2. Comparison of the Salvia Total Phenolic Acids fingerprint profiling on Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 150 mm, 1.9 µm and 2.1 × 100 mm columns.

5991-7559EN

It's not only about efficiency



$$R_s = \frac{\sqrt{N}}{4}\frac{(\propto -1)}{\propto}\frac{k}{(k+1)}$$

To increase resolution:

- Increase retention (*k*)
- Change selectivity (*α*)
- Increase efficiency (*N*)

- Change particle to impact *N*, smaller diameter and SPP for higher *N*
- Change mobile phase or column chemistry to impact *k* and α

Selectivity is also important

Best all around	Best for high pH mobile phases	Best for alternative selectivity	Best for more polar compounds
InfinityLab Poroshell 120 **EC-C18** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell **HPH-C18** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell 120 **PFP** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell 120 **HILIC** 1.9 µm, 2.7 µm, 4 µm
InfinityLab Poroshell 120 **EC-C8** 1.9 µm, 2.7 µm, 4 µm			
InfinityLab Poroshell 120 **Phenyl-Hexyl** 1.9 µm, 2.7 µm, 4 µm			

A range of different chemistries provides the selectivity options to develop methods quickly

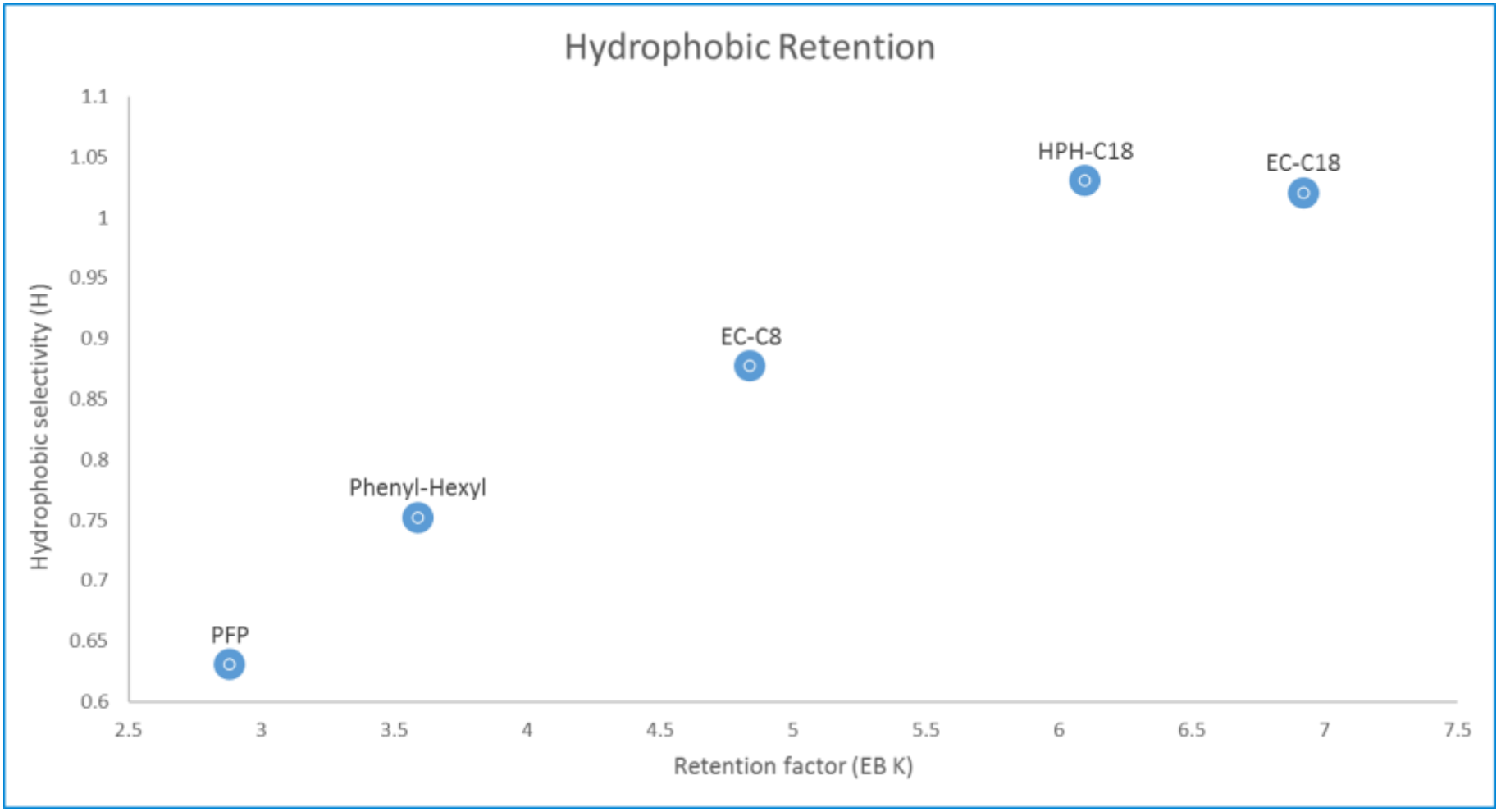
Characterizing selectivity
Tanaka and Hydrophobic subtraction model (HSM)

Tanaka	HSM	Parameter	Details
α CH2	H	Hydrophobicity	Separation based on differences in analyte hydrophobicity
α T/O	S*	Steric interaction	Separation based on differences in analyte shape
α C/P	A	Hydrogen-bond acidity	Separation based on hydrogen bonding by basic analytes
	B	Hydrogen-bond basicity	Separation based on hydrogen bonding by acidic analytes
α $B/P_{(pH\ 2.7)}$	$C_{(pH\ 2.8)}$	Ion-exchange capacity	Separation based on ion exchange by analyte at pH <3
α $B/P_{(pH\ 7.6)}$	$C_{(pH\ 7.0)}$	Ion-exchange capacity	Separation based on ion exchange by analyte at pH ≥ 7
k PB	EB	Hydrophobic retention	Retention of a neutral analyte

HSM also features the F_s factor to describe the similarity of two column selectivities. A small F_s indicates that two columns are very similar, while a large factor indicates that two columns are very different.

Further details at: http://www.hplccolumns.org/

Retention and selectivity



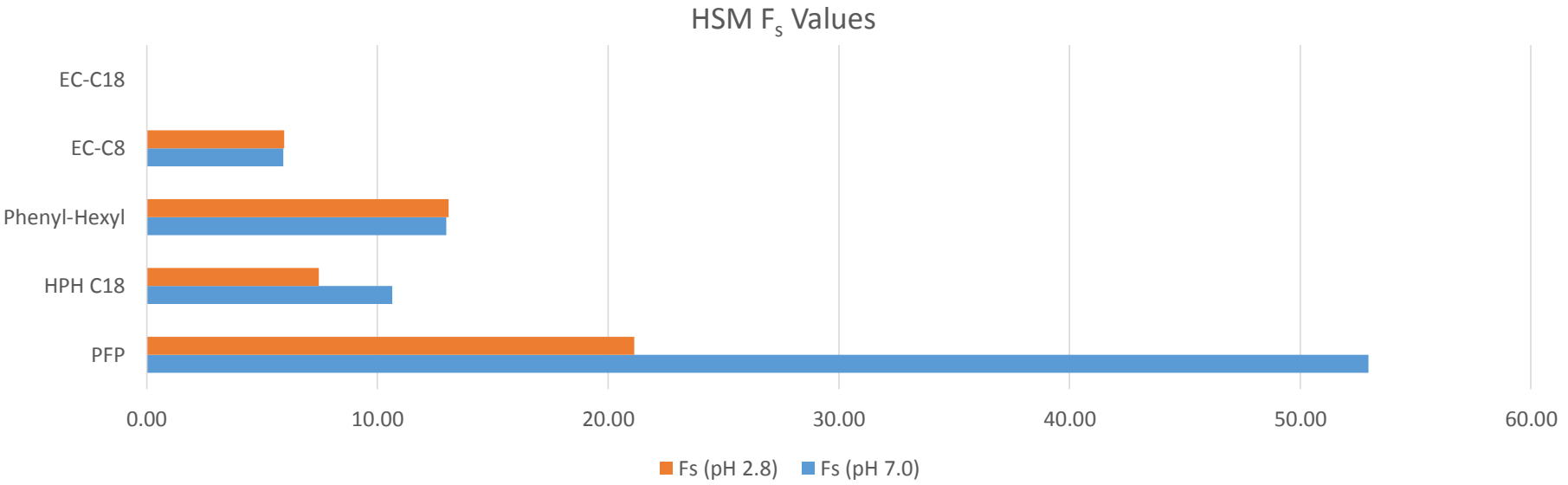
HSM Fs values for InfinityLab Poroshell 120

$$F_s = \{[12.5(H_2 - H_1)]^2 + [100(S_2^* - S_1^*)]^2 + [30(A_2 - A_1)]^2 + [143(B_2 - B_1)]^2 + [83(C_2 - C_1)]^2\}^{\frac{1}{2}}$$

Chemistry	H	S*	A	B	C (pH 2.8)	C (pH 7.0)	EB K	Fs (pH 7.0)	Fs (pH 2.8)
EC-C18	1.02	0.008	-0.13	-0	0.161	0.123	6.9	0.00	0.00
EC-C8	0.88	0.011	-0.23	0.023	0.127	0.09	4.8	5.92	5.96
HPH C18	1.03	0.005	-0.14	-0.01	0.073	-0.004	6.1	10.65	7.46
Phenyl-Hexyl	0.75	-0.08	-0.39	0.018	0.136	0.14	3.6	12.99	13.08
PFP	0.63	-0.06	-0.46	0.015	-0.038	0.741	2.9	52.96	21.14

Reversed-phase only so HILIC not included

Hydrophobic Subtraction Model (HSM) Data provided by Dwight Stoll



Benzodiazepines

Results and Discussion

Figure 2 shows the separation of eight bezodiazepines on a Poroshell 120 EC-C18, 2.1 × 150 mm, 1.9 µm column. All compounds were baseline-resolved, with a minimum resolution of 2.2, in 5 minutes. The benzodiazepines were difficult to separate because they are structurally very similar (Figure 1). However, the Poroshell 1.9 µm column had sufficient efficiency and resolving power to successfully separate this sample.

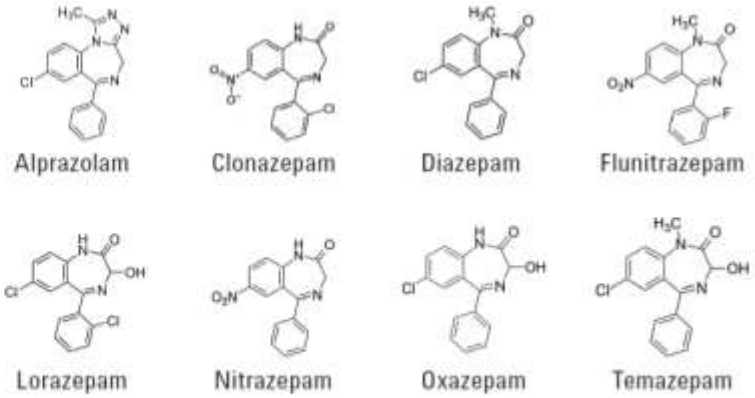


Figure 1. Compounds of interest.

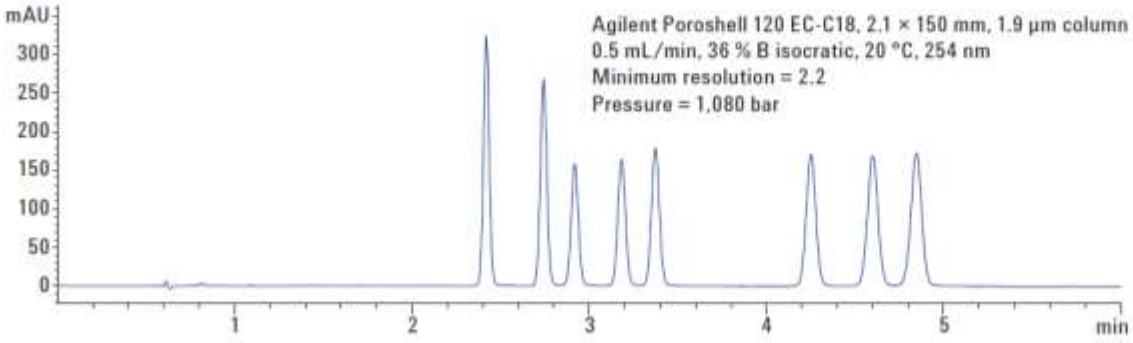
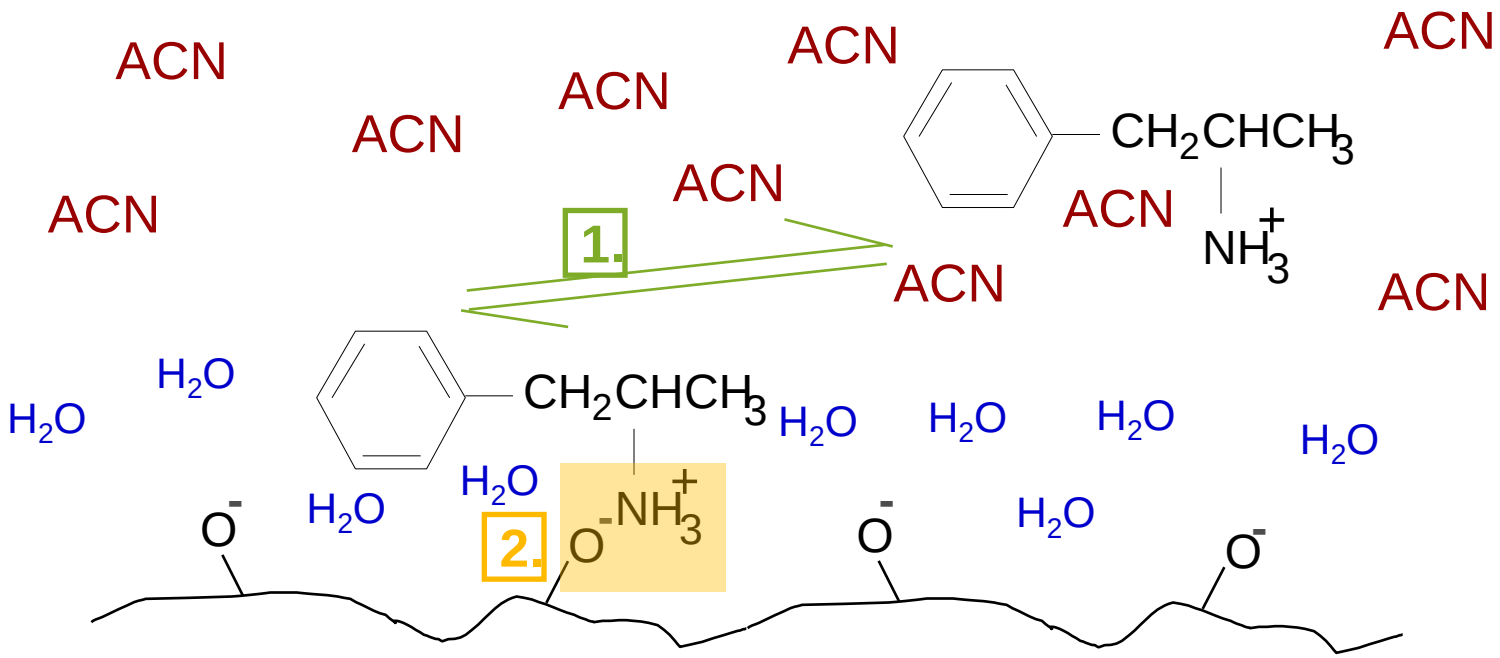


Figure 2. Separation of benzodiazepines on an Agilent Poroshell 120 EC-C18, 1.9 µm column.

5991-7539EN

HILIC

Hydrophilic interaction liquid chromatography



1. Partitioning in and out of adsorbed water layer
2. Ion exchange with silanols

HILIC separation
Free amino acids

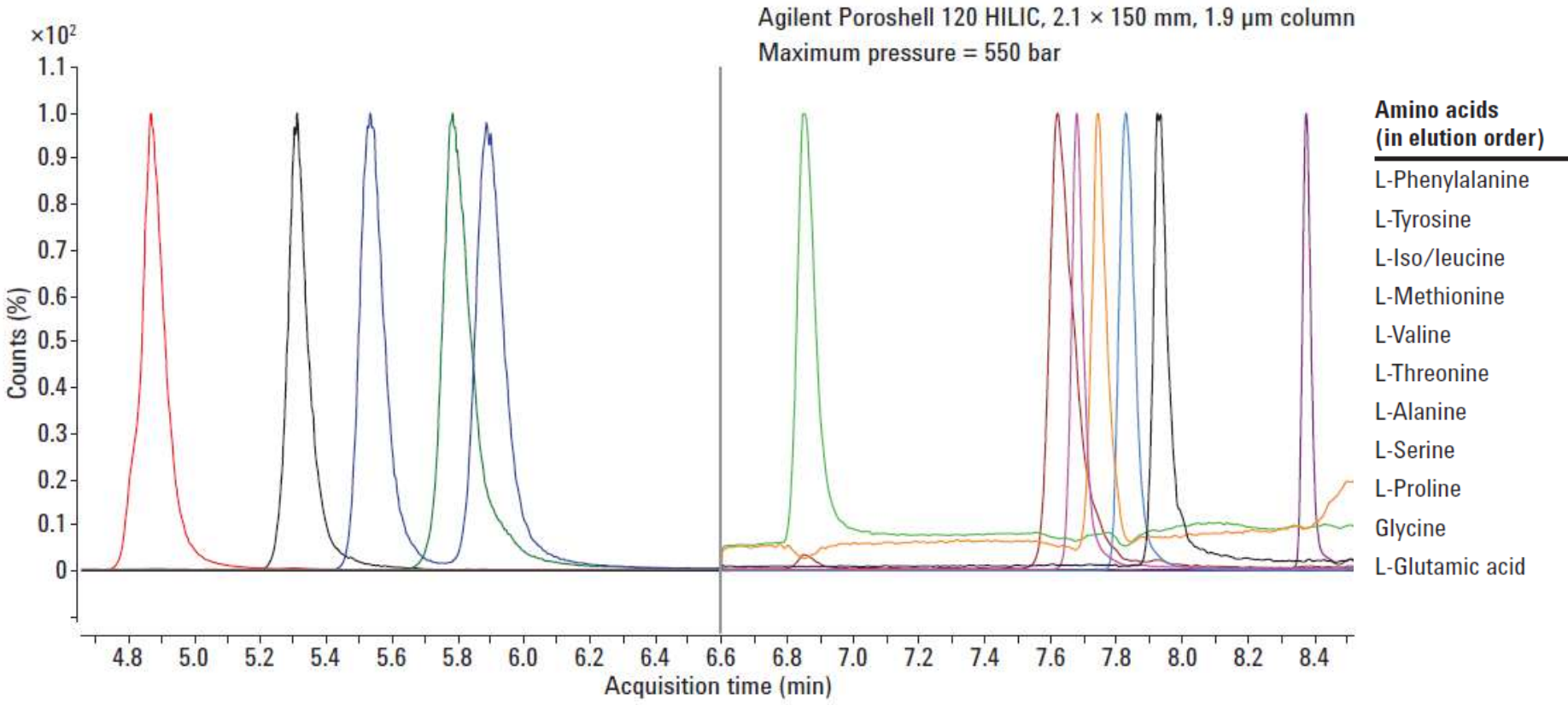


Figure 1. Separation of 12 free amino acids on an Agilent Poroshell 120 HILIC 1.9 µm column.

5991-7541EN

Free amino acids
Separation of isobaric compounds

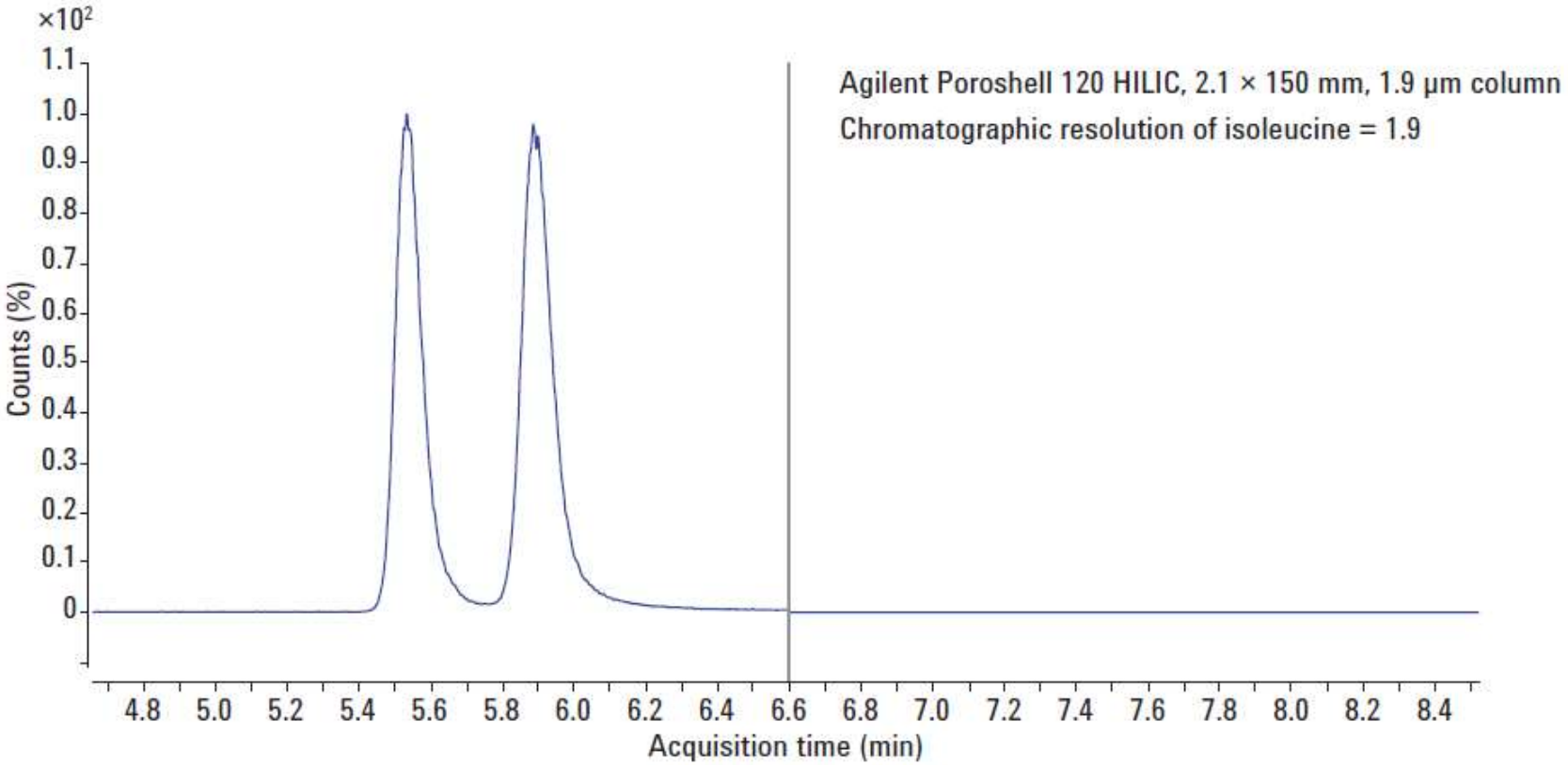


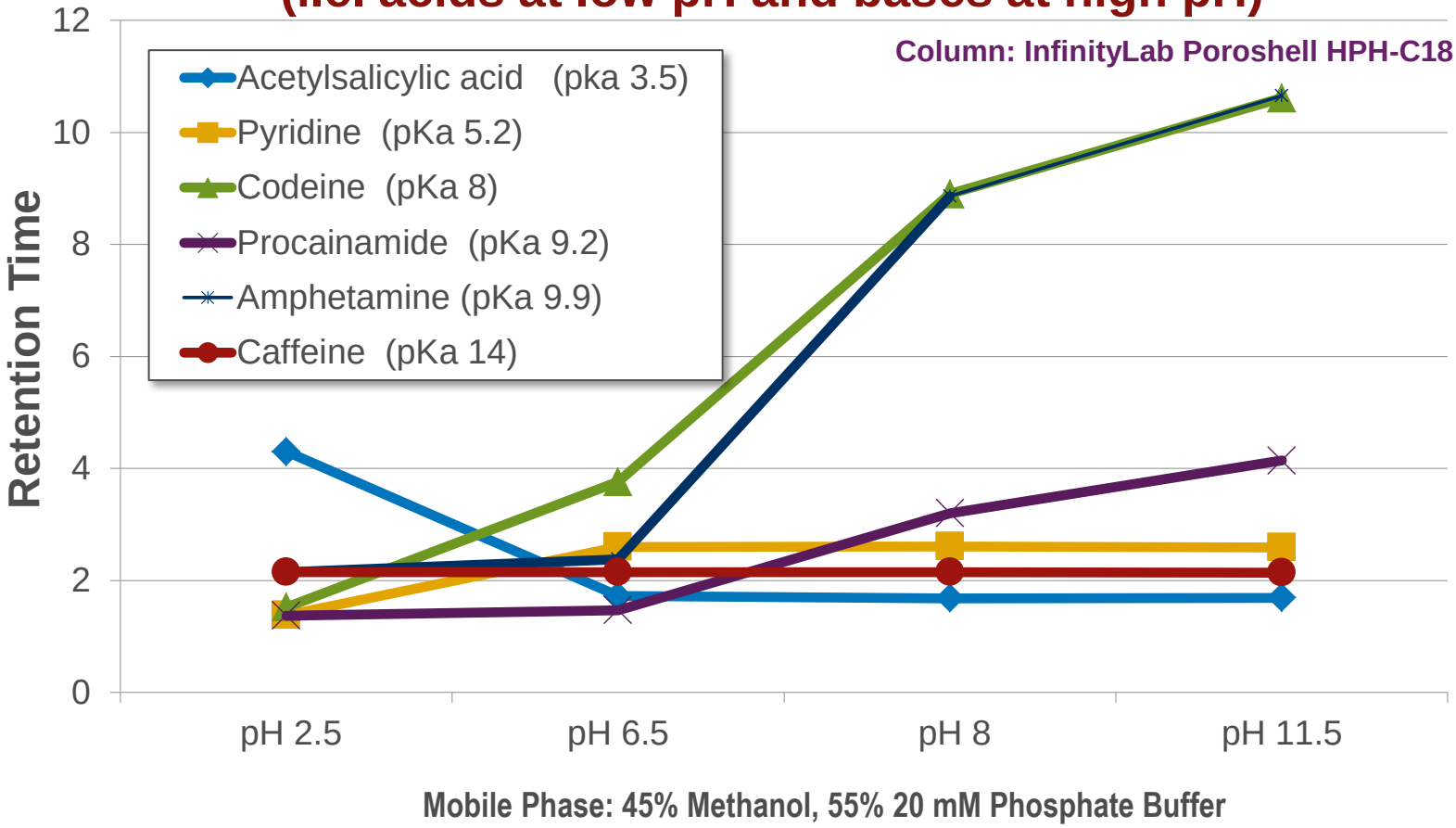
Figure 2. Separation of isobaric leucine and isoleucine on an Agilent Poroshell 120 HILIC, 1.9 µm column.

5991-7541EN

Impact of mobile phase pH on selectivity

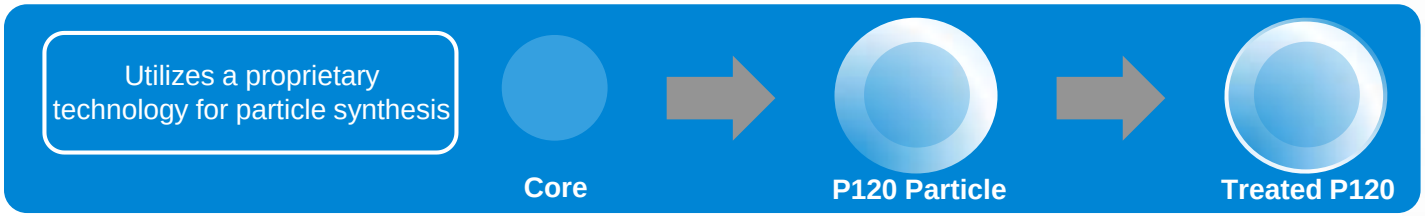
Change in retention with mobile phase pH

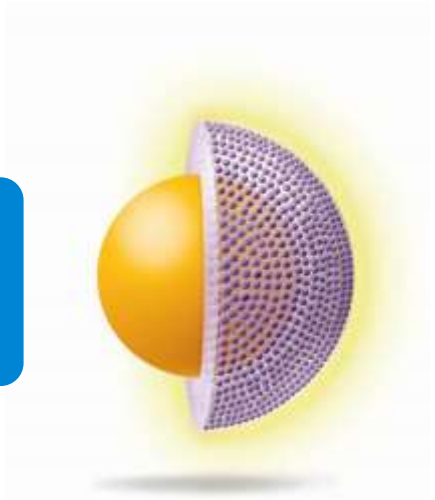
More retention for non-charged analytes
(i.e. acids at low pH and bases at high pH)



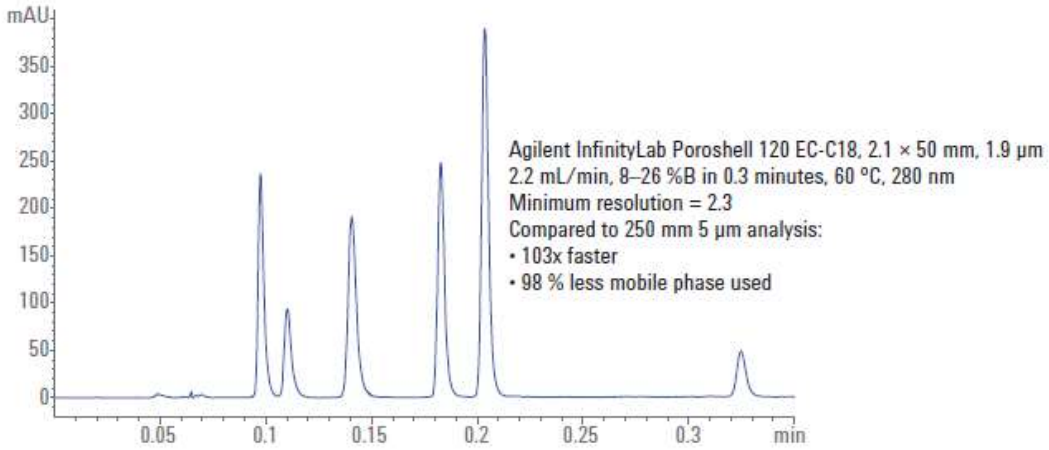
Approaches for longer lifetime at high pH

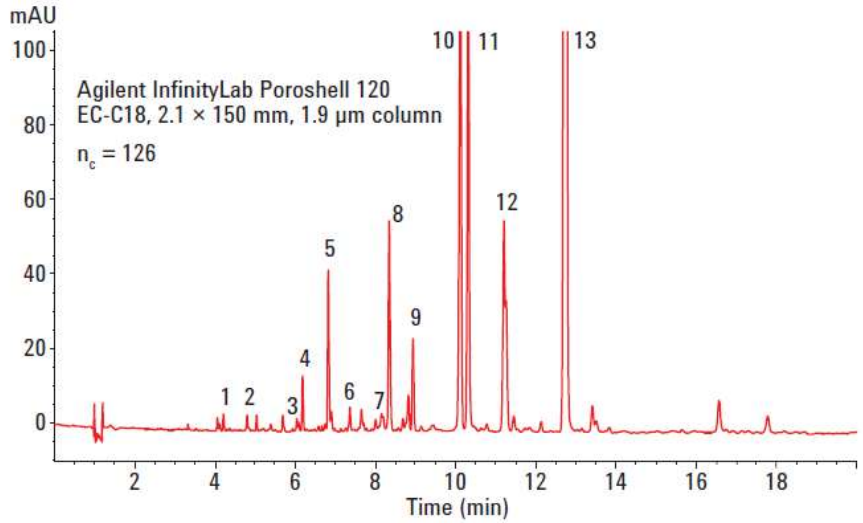
Approach	Comments
Totally porous silica-hybrid particles	Do not have the efficiency of superficially porous particles
Bonding chemistry on superficially porous silica particles	Do not have the lifetime of silica-hybrid particles
Integration of organic compound into the porous layer of SPP (prior to bonding)	Combine the advantages of silica-hybrid and superficially porous particles





Value of UHPLC





Improve throughput and decrease cost-per-sample

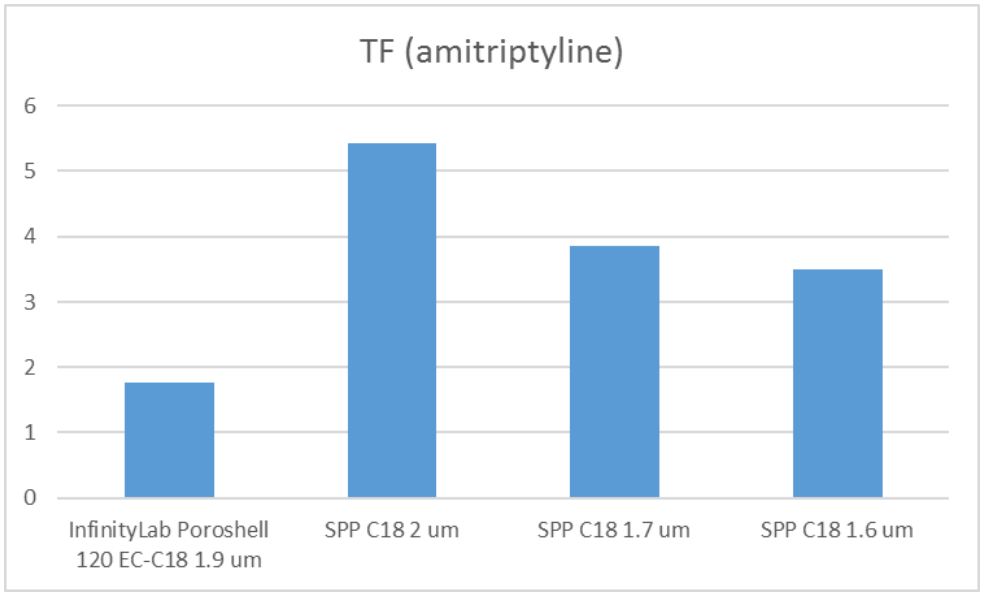
Increase the accuracy and precision of analysis results and reduce re-work

Issues getting the best value from UHPLC

Issue	Consequence	Impact on value
Columns give poor peak shape (tailing)	Decreased resolution leading to lower accuracy and precision of analysis results	Increased costs due to re-work
Columns generate very high pressure	Cannot be run at optimum flow rate leading to slower runs or lower resolution	Increased costs due to lower throughput or increase in re-work
Columns cannot maintain high efficiency under UHPLC conditions	Need to be replaced often	Increased costs due to column purchase, disruption and re-work

Peak shape

Issue	Consequence	Impact on value
Columns give poor peak shape (tailing)	Decreased resolution leading to lower accuracy and precision of analysis results	Increased costs due to re-work

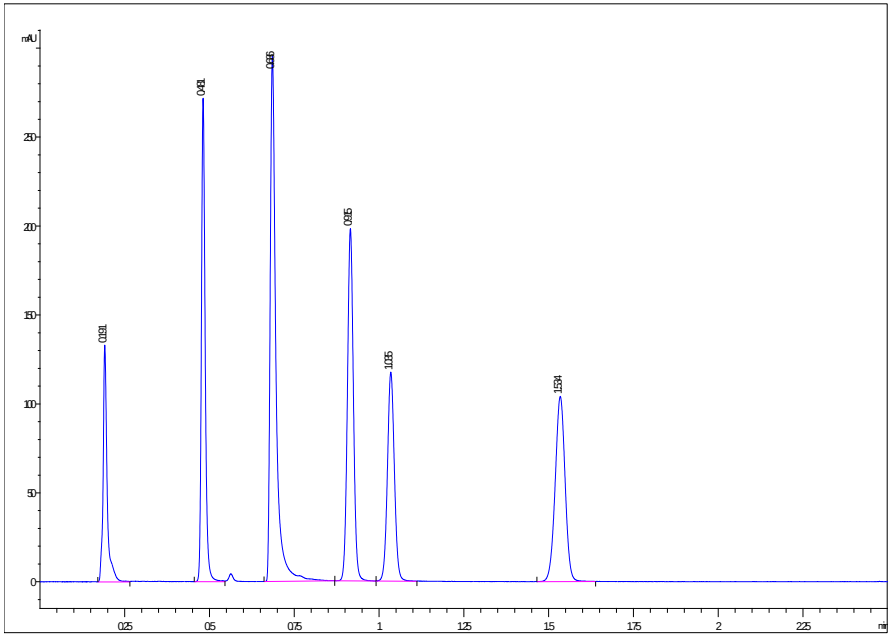


Superior peak shapes improve the accuracy and precision of your analytical results

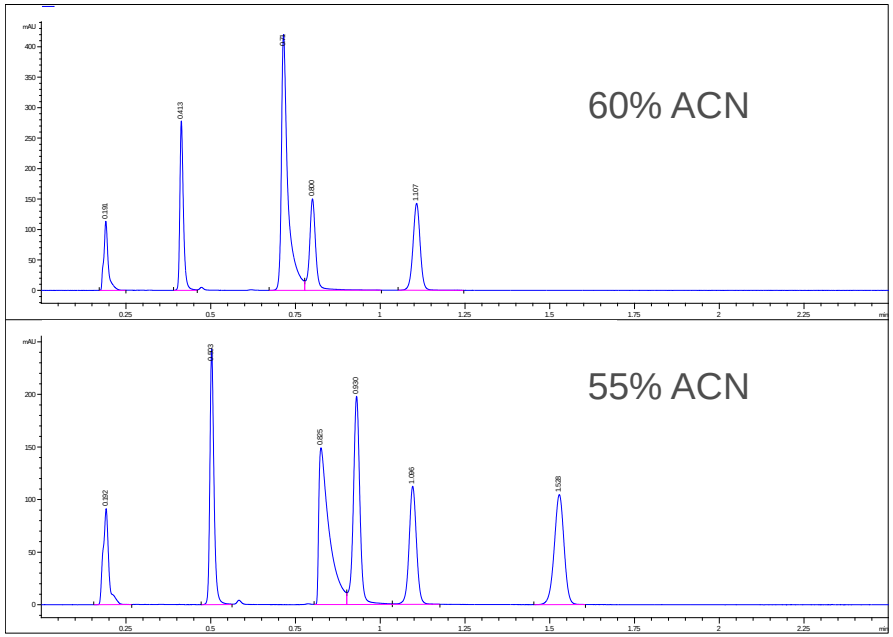
1290 LC with ULD kit, 2.1 x 50 mm columns, 40% 20 mM Sodium Phosphate pH 7, 55 or 60% Acetonitrile, 0.5 mL/min, 0.5 uL, 25C, 254 nm, 80 Hz

What superior peak shape looks like

Poroshell 120 EC-C18 1.9 µm



SPP 1.7 µm

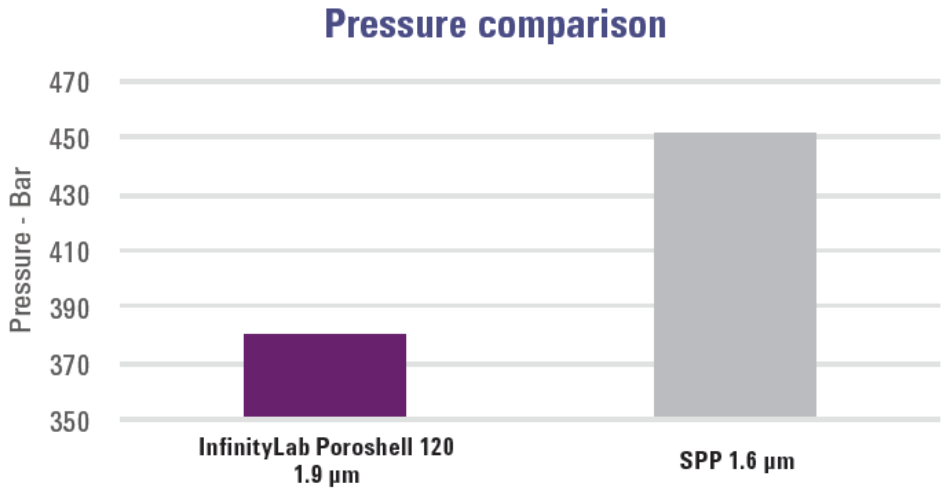


Low peak tailing, increased resolution

1290 LC with ULD kit, 2.1 x 50 mm columns, 40% 20 mM Sodium Phosphate pH 7, 55 or 60% Acetonitrile, 0.5 mL/min, 0.5 uL, 25C, 254 nm, 80 Hz

Manageable pressure

Issue	Consequence	Impact on value
Columns generate very high pressure	Cannot be run at optimum flow rate leading to slower runs or lower resolution	Increased costs due to lower throughput or increase in re-work

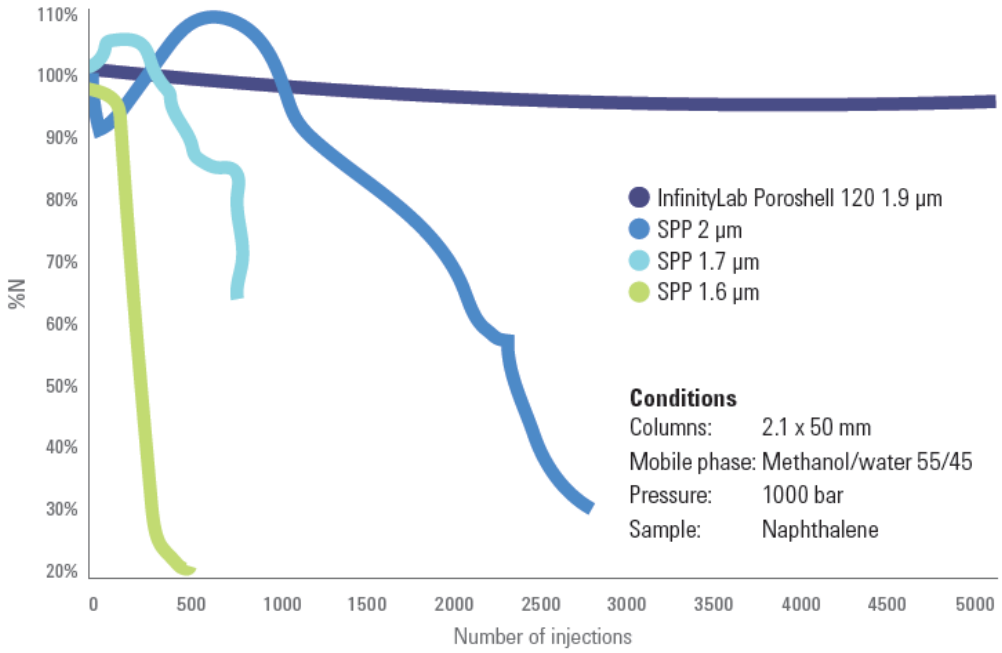


Manageable pressure allows you to use your columns at optimum flow rate and/or use longer columns without exceeding the pressure rating of your UHPLC instruments

1290 LC with ULD kit, 2.1 x 50 mm columns, 40% 20 mM Sodium Phosphate pH 7, 55 or 60% Acetonitrile, 0.5 mL/min, 0.5 uL, 25C, 254 nm, 80 Hz

Longest column lifetime

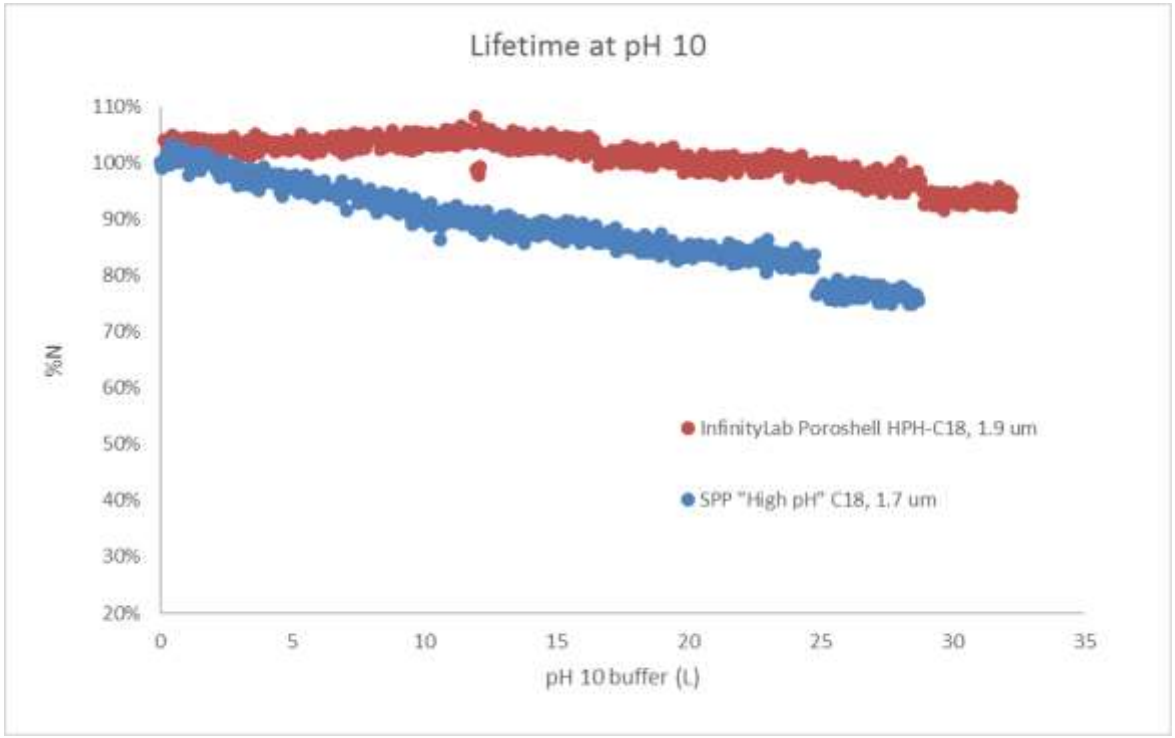
Issue	Consequence	Impact on value
Columns cannot maintain high efficiency under UHPLC conditions	Need to be replaced often	Increased costs due to column purchase, disruption and re-work



Longer column lifetime reduces your costs for column replacement and the associated disruption and re-work

Column lifetime at high mobile phase pH

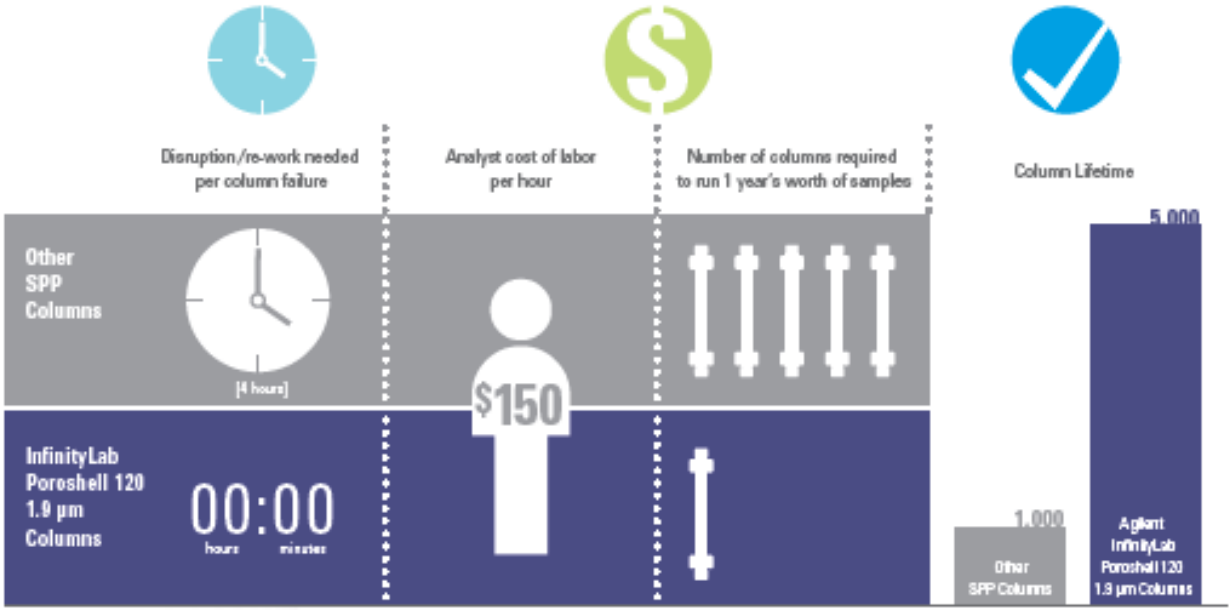
- Working at high mobile phase pH provides additional selectivity options – but the column must be stable under these conditions



Compatibility with high pH mobile phases means that additional selectivity options are available - allowing you to improve the accuracy and precision of your analytical results

Columns: 2.1 x 50 mm, Isocratic pH 10, 50°C 10 mM Ammonium Bicarbonate, 0.4 mL/min, Sample: Butyl benzene

Economic value of robust UHPLC columns



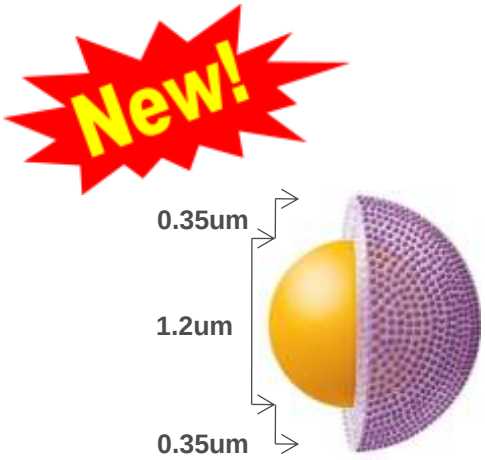
	InfinityLab Poroshell 120 EC-C18 1.9 um	SPP C18 1.7 um	Economic value
Number of analyses	5000		
Labour rate	$150/hour		
Disruption due to column failure	2-hours		
Rework due to column failure	2-hours		
Column price	$825	$765	
Column lifetime	5000 inj.	1000 inj.	
Column purchase costs	$825	$3825	$3000
Disruption costs	$0	$1200	$1200
Rework costs	$0	$1200	$1200
		TOTAL	$5400

- Long lifetime not only reduces column spend – fewer columns are needed for the same amount of work – but also reduces costs due to the disruption caused and re-work required when columns fail
- This represents a significant economic value to your laboratory

Getting the best value from UHPLC

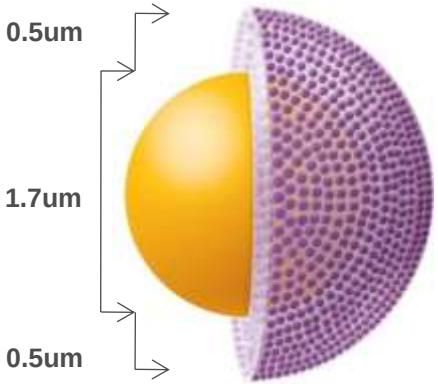
Feature	Advantage	Economic value
High purity silica, quality novel chemistries	Superior peak shape	Decreased costs due to re-work
Robust superficially porous 1.9 um particles, optimized column loading	Manageable pressure	Decreased costs due to lower throughput or increase in re-work
Robust superficially porous 1.9 um particles, optimized column loading, novel chemistries	Long column lifetime	Decreased costs due to column purchase, disruption and re-work

Scalable family of particles



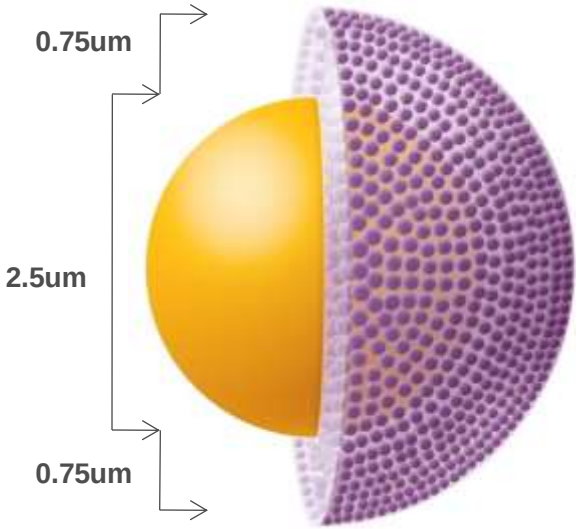
InfinityLab Poroshell 120
1.9 µm

Highest UHPLC performance



InfinityLab Poroshell 120
2.7 µm

UHPLC performance at lower pressure



InfinityLab Poroshell 120
4 µm

Improved HPLC performance

www.agilent.com/chem/discoverporoshell

The advantage of a scalable family of particles
Aromatic acids

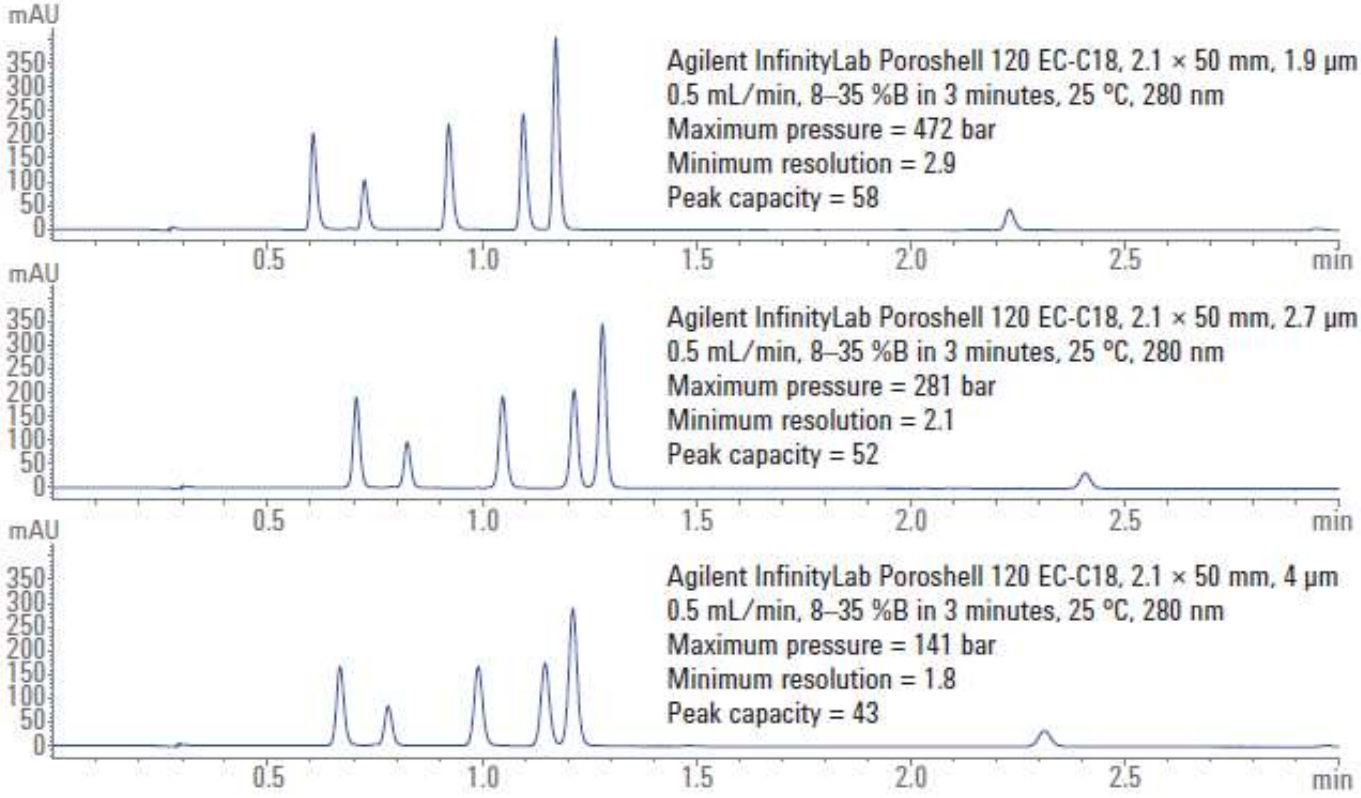
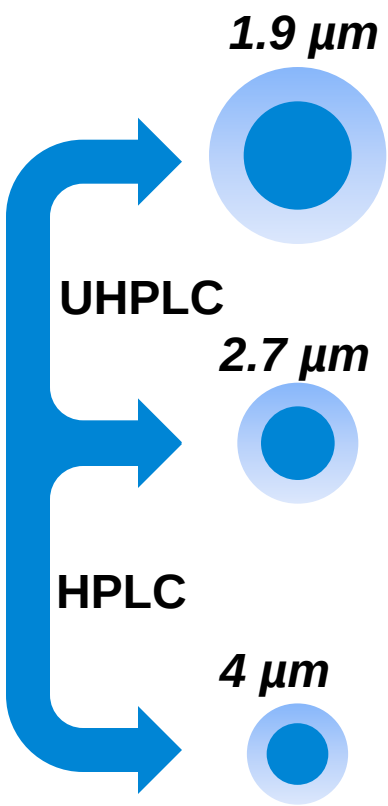


Figure 3. Similar selectivity among Agilent InfinityLab Poroshell particle sizes allows analysts to choose their column configuration based on instrument pressure limits or method performance requirements without needing to do additional method development.

5991-7518EN

An SPP column for everyone



Performance

Highest UHPLC performance
Pressure rating: 1300 bar
Typical pressure: Similar to sub-2 µm totally porous
Efficiency: ~120% of sub-2 µm totally porous

UHPLC performance at lower pressure
Pressure rating: 600 bar
Typical pressure: 50% of sub-2 µm totally porous
Efficiency: ~90% of sub-2 µm totally porous

Improved HPLC performance
Pressure rating: 600 bar
Typical pressure: Often < 200 bar
Efficiency: ~200% of 5 µm totally porous

Use all the instruments in your laboratory

Instruments		We recommend…
	UHPLC only **Maximum pressure:** High (> 600 to 1000+ bar) **Dispersion volume:** Very low	InfinityLab Poroshell 120 1.9 µm InfinityLab Poroshell 120 2.7 µm
	HPLC and UHPLC **Maximum pressure:** Low to high (400 to 1000+ bar) **Dispersion volume:** Medium to very low	InfinityLab Poroshell 120 2.7 µm InfinityLab Poroshell 120 4 µm
	HPLC only **Maximum pressure:** Low to mid (400 to 600 bar) **Dispersion volume:** High to low	InfinityLab Poroshell 120 4 µm InfinityLab Poroshell 120 2.7 µm

Summary

- UHPLC is used either for very fast or very high resolution separations
- High efficiency is a key requirement for UHPLC and high pressure a key consequence
- Minimized instrument dispersion volume is key for successful UHPLC
- < 2 um SPP provide very high efficiency separations
- Methods can be developed quickly and easily with a range of chemistries, including phases for high pH work
- UHPLC columns providing a unique combination of superior peak shape manageable pressure and long lifetime represents significant economic value to your lab
- You can select the best column for your needs/instruments from a scalable family of particles

Find out more

Learn more
www.agilent.com/chem/discoverporoshell

Get support
www.agilent.com/chem/cstechsupport

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- **Europe:** info_agilent@agilent.com
- **Asia Pacific:** inquiry_lsca@agilent.com

Ordering details

Size (mm)	EC-C18	EC-C8	Phenyl-Hexyl	HPH-C18	PFP	HILIC
3.0 x 150	693675-302	693675-306	693675-312	693675-502	693675-308	693675-301
3.0 x 100	695675-302	695675-306	695675-312	695675-502	695675-308	695675-301
3.0 x 50	699675-302	699675-306	699675-312	699675-502	699675-308	699675-301
2.1 x 150	693675-902	693675-906	693675-912	693675-702	693675-408	693675-901
2.1 x 100	695675-902	695675-906	695675-912	695675-702	695675-408	695675-901
2.1 x 50	699675-902	699675-906	699675-912	699675-702	699675-408	699675-901

Size (mm)	EC-C18	EC-C8	Phenyl-Hexyl	HPH-C18	PFP	HILIC
3.0 x 5	823750-940	823750-941	823750-943	823750-945	823750-942	823750-944
2.1 x 5	821725-940	821725-941	821725-943	821725-945	821725-942	821725-944

All InfinityLab Poroshell 120 1.9 µm columns are supplied with a pre-programmed Column ID

Column ID

Usability, traceability and security

- All InfinityLab Poroshell 120 1.9 µm columns are shipped with pre-installed and pre-programmed Column ID

Column ID

Clip that holds column ID to column

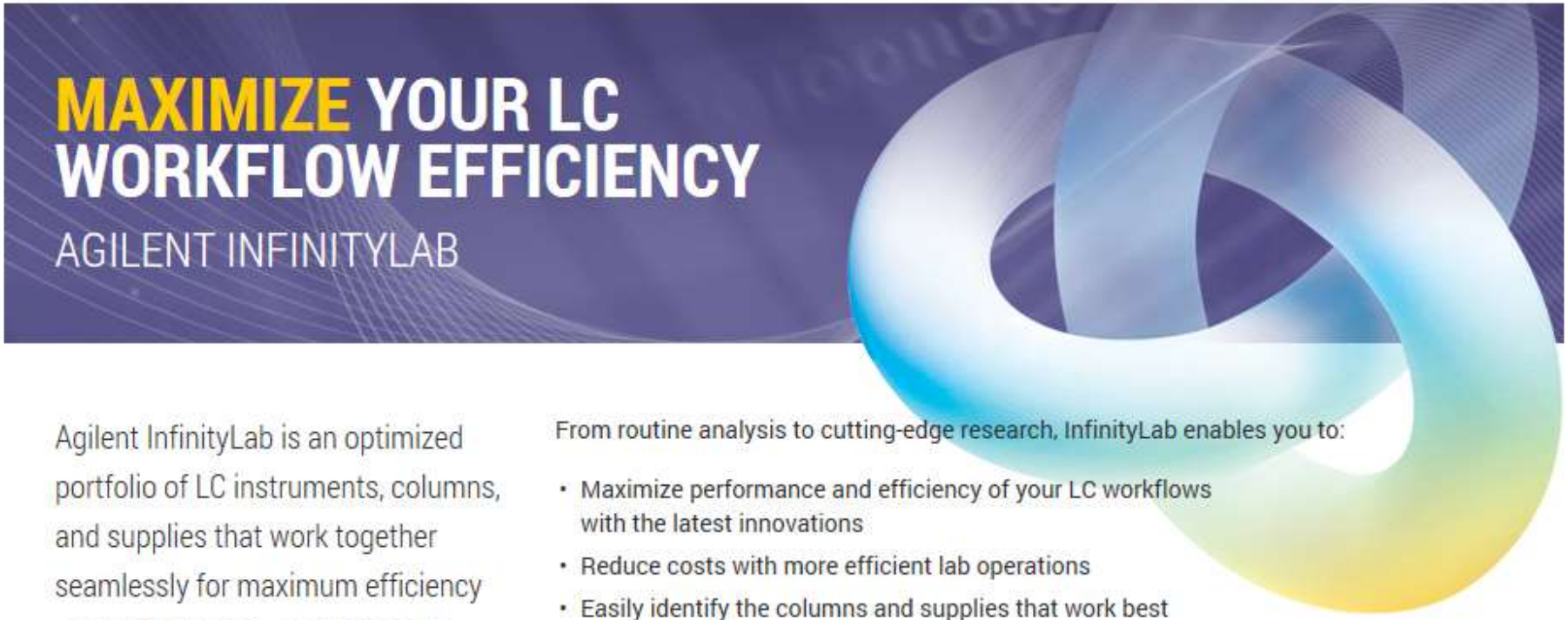
Understand key details and use of your column

Field	Example
Description	Poroshell EC-C18
Length [mm]	100
Diameter [mm]	4.6
Particle size [µm]	2.7
Maximum pressure [bar]	600
Number of injections	[counter]
Product number	695975-902T
Serial number	USABC12345
Batch number	B12345
Maximum temperature [°C]	60
Maximum measured temperature [°C]	[updated from instrument]
Minimum pH	2.0
Maximum pH	8.0
Void volume [mL]	1.00
First injection date	[updated from instrument]
Recent injection date	[updated from instrument]

- **Usability**
  - Easily find column details
- **Traceability**
  - Always know exactly which column is/was installed
- **Security**
  - Protect against the use of methods incompatible with the column

Agilent InfinityLab

www.agilent.com/chem/InfinityLab



Agilent InfinityLab is an optimized portfolio of LC instruments, columns, and supplies that work together seamlessly for maximum efficiency and performance—regardless of application area.

From routine analysis to cutting-edge research, InfinityLab enables you to:

- Maximize performance and efficiency of your LC workflows with the latest innovations
- Reduce costs with more efficient lab operations
- Easily identify the columns and supplies that work best with your Agilent InfinityLab LC Series instruments

Combined with Agilent OpenLAB software and Agilent CrossLab Services, Agilent provides you with end-to-end solutions and support to ensure the best analytical outcomes.