Optimization of HPLC Instrumentation for Use with Poroshell 120 Columns

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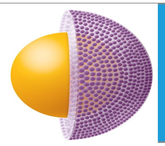

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Introduction

Superficially porous HPLC columns, such as Poroshell 120 are able to deliver similar performance to sub-two micron materials at substantially lower pressures. While modern HPLC instrumentation and data systems are able to capture the benefits of these particles, attention to the instrumentation configuration is important if optimal results are desired. Configuration should be optimized with regard to the speed with which peaks pass through the detector and the small peak volumes produced in the system. Peak volumes of 50 to 75 % lower volume as compared to conventional 5 and 3.5 micron particle filled columns of similar size are found. The most relevant system parameters are detector response time, connecting tubing volume and flow cell volume. Comparisons of chromatographic performance will be shown in which each of these parameters is optimized and de-optimized. The combined effect of using all three in an optimized and a de-optimized mode will emphasize the results. Guidelines will be given to help the chromatographer achieve best results.

Optimize the Whole Experiment

System Optimization

1. Optimize the data collection rate of the detector. Set the data collection rate to the fastest setting such that signal to noise (S/N) is not adversely affected.
2. Choose the flow cell carefully. The standard flow cell on the Agilent 1200 has a volume of 13 µl. This may diminish the performance achievable using Poroshell 120 columns. Smaller volume flow cells such as the semi micro (6 mm/5 µl)or micro (3 mm/2 µl) are recommended for best performance.
3. Minimize Extra Column Volume. Use the shortest length of tubing possible for all connections. Use Red (0.12 mm id) tubing instead of Green (0.17 mm) as it has only half of the volume of the larger green tubing. Use proper fittings for all connections. Make zero dead volume connections. These will ensure best peak shape.

Chromatography Optimization

1. Optimize the flow rate to achieve the desired efficiency (N) or peak capacity (Pc).
2. If adapting an older method to a new Poroshell 120 column properly scale the gradient and injection volume to the new smaller column to quickly transfer the method and avoid overloading.
3. Minimize sample dispersion in the column.
   1. Use an injection solvent that is weaker than the mobile phase, especially when using an isocratic method.
   2. Gradients can minimize dispersion but be aware of possible effects.

This work was performed on an Agilent 1200 RRLC

It included a G1312 B Pump, a G1367A Autosampler and a G1315C DAD SL Detector. Data was collected and processed using ChemStation version B 3.01

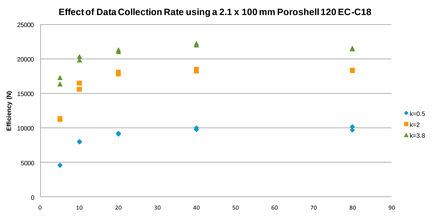
Optimization of Data Collection

Optimize Detector Speed

Optimize detector settings by adjusting the scan rate and/or the time constant to the fastest possible settings such that signal-to-noise (S/N) is not adversely affected.

The Peakwidth control in ChemStation enables you to select the peak width(response time) for your analysis. The peak width (as defined in the ChemStation software) is the width of a peak at half height. Set the peak width to the narrowest expected peak in your sample. With Poroshell 120 column expect narrow peaks, similar to those generated with sub 2 micron columns. Set the detector to the fastest setting, then to the second fastest setting and evaluate if the signal to noise levels are different.

Data Collection Rate Importance



Column part number 695775-902

1 ul QC Mix, Uracil, Phenol (k=0.5), 4-Chloronitrobenzene(k=2), Napthalene(k=3.8) 55% MeCN 45 % Water 0.55 ml/min micro flow cell

For best results when using small columns, collect data at 40 Hz.

Slower data collection is practical when analytes retain longer. (k'> 4)

Choice of Flow Cell

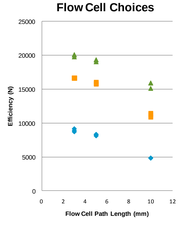
Flow Cells are an integral part of HPLC instrumentation. The tendency is to use a larger flow cell in order to more easily detect the compounds of interest is common. However, as shown in this work, they can also be a source of extra column volume. Choose the flow cell carefully.

While detector speed can compensate for excessive flow cell dispersion, an appropriate flow cell should be used,

The volume of a Standard flow cells for an Agilent 1100 or 1200 system is 10 µL.

For best results, replace standard flow cells with 5 µL flow cells (2 µL when using 2.1 mm ID columns)

Flow Cell Choices with a 2.1 x100 mm Poroshell 120 EC-C18



30 % loss of efficiency with a 10 mm standard flow cell With 2.1 mm columns, it is best to use a 3 mm flow cell.

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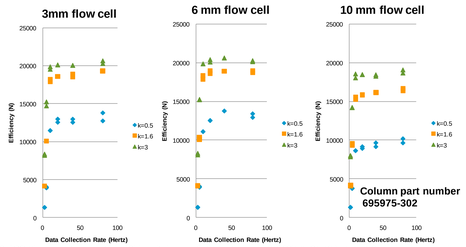
1 ul QC Mix, Uracil, Phenol (k=0.5), 4-Chloronitrobenzene(k=2), Napthalene (k=3.8) 55% MeCN 45 % Water 0.55 ml/min micro flow cell

Data Collection Rate Combined with Flow Cell Choice

Larger Flow Cells, used at Fast Data Collection speeds can be used with Larger Columns as shown with this 3 x100 mm Poroshell 120 EC-C18 Column

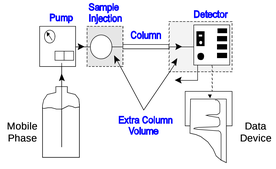


2 ul QC Mix, Uracil, Phenol (k=0.5), 4-Chloronitrobenzene(k=2), Napthalene(k=3.8) 60% MeCN 40 % Water 0.8 ml/min micro flow cell

Flow Cells	Path Length	Volume	Part Number
Standard (with RFID tag)	10 mm	13 µl	G1315-60022
Semi-micro (with RFID tag)	6 mm	5 µl	G1315-60025
Micro (with RFID tag)	3 mm	2 µl	G1315-60024

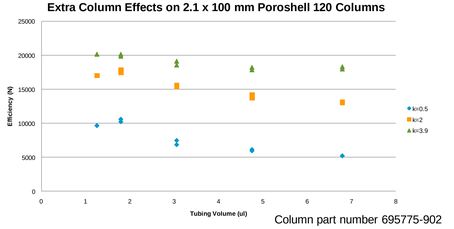
Minimize Extra Column Volume

Instrument Contributes to Column Performance



Extra Column Volume = sample volume + connecting tube volume + fitting volume + detector cell volume

2.1 x 100 mm Poroshell 120 Columns (early eluters are more severely affected by extra column broadening)



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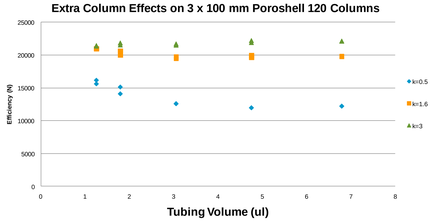
3 x 100 mm Poroshell 120 Columns

(early eluting compounds are less effected by extra column volume than with 2.1 x 100 mm Poroshell 120 columns)



Proper Column Connections

Use of 0.12 mm Tubing instead of 0.17 mm Tubing This reduces extra column volume by half.

Inside Diameter (mm)	Length (mm)	Material	Color	Connections	Part Number	Volume (ul)
0.12	180	SS	Red	1 end pre-swaged	G1313-87304	2.0
0.12	280	SS	Red	1 end pre-swaged	01090-87610	3.2
0.12	105	SS	Red	1 end pre-swaged	01090-87611	1.2
0.12	150	SS	Red	pre-swaged	G1315-87312	1.7
0.12	105	SS	Red	Without fittings	5021-1820	1.2
0.12	150	SS	Red	Without fittings	5021-1821	1.7
0.12	280	SS	Red	Without fittings	5021-1822	3.2
0.12	400	SS	Red	Without fittings	5021-1823	4.5
0.17	180	SS	Green	1 end pre-swaged	G1313-87305	4.1
0.17	280	SS	Green	1 end pre-swaged	01090-87304	6.4
0.17	130	SS	Green	1 end pre-swaged	01090-87305	2.9
0.17	90	SS	Green	1 end pre-swaged	G1316-87300	2.0
0.17	105	SS	Green	Without fittings	5021-1816	2.4
0.17	150	SS	Green	Without fittings	5021-1817	3.4
0.17	280	SS	Green	Without fittings	5021-1818	6.4
0.17	400	SS	Green	Without fittings	5021-1819	9.1

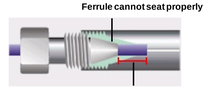
USE LOWER VOLUME RED TUBING WHEN POSSIBLE

GREEN TUBING HAS 2 x VOLUME OF RED TUBING FOR EQUAL LENGTH

Peak tailing/fronting What Happens If the Connections Poorly Made ?

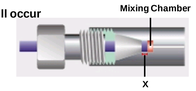
Wrong … too long

Ferrule cannot seat properly



If Dimension X is too long, leaks will occur

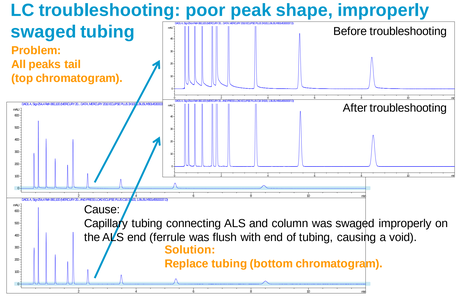
Wrong … too short



If Dimension X is too short, a dead-volume, or mixing chamber, will occur

LC troubleshooting: poor peak shape, improperly swaged tubing

Problem:
All peaks tail (top chromatogram).



Cause:
Capillary tubing connecting ALS and column was swaged improperly on the ALS end (ferrule was flush with end of tubing, causing a void).

Solution:
Replace tubing (bottom chromatogram).

Stainless Steel and Polymer Fittings

Which type is used and when?

Stainless Steel (SS) fittings are the best choice for reliable high pressure sealing

Agilent uses Swagelok type fittings with front and back ferrules, which give best sealing performance throughout our LC system (use this on the instrument connections, i.e. valves, heaters etc)

PEEK (<400 bar system pressure) fittings are ideal where:

- **Connections are changed frequently, i.e. connecting columns**
- **Bio-compatibility is needed**
- **Pressure is less critical**

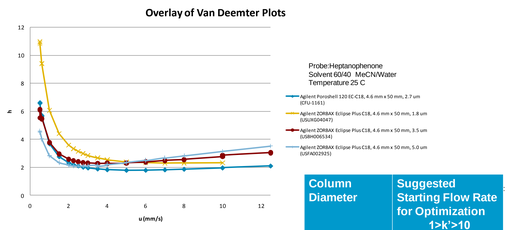
Polyketone fittings can be used up to 600 bar

Use this fitting on column connections with Poroshell 120 (PN 5042-8957)

Some typical column connectors shown here

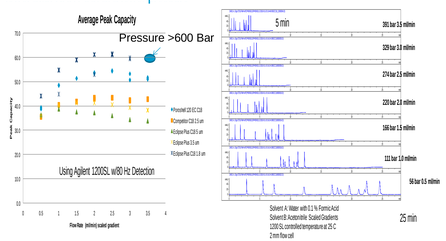
Optimize Flow Rate

Optimal Flow Rate for Poroshell Faster than for 5 or 3.5 micron Columns it Replaces



Column Diameter	Suggested Starting Flow Rate for Optimization 1>k'>10
4.6 mm	2 ml/min
3.0 mm	0.85 ml/min
2.1 mm	0.42 ml/mi

Peak Capacity of Poroshell 120 is slightly lower than RRHT but at a lower pressure



Conclusion

1. **Poroshell 120 columns can achieve similar efficiencies as sub 2 micron columns with substantially less pressure.**
2. **Excellent results can be achieved achieved on Agilent 1200 RRLC or 1290 Infinity**
3. **In order to achieve best performance**
   1. **Optimize data collection rate (40 Hz detector with fast response time)**
   2. **Use the smallest flow cell that you have (3mm micro flow cell works best when using small columns).**
   3. **Minimize extra column volume: flow cell and connecting tubing are the biggest contributors, but needle seats should be replaced with smaller volume seats. Red tubing has half the volume of green tubing.**
   4. **Take care to make proper connections.**
   5. **Optimize flow rates for best performance. Start with flow rate 2 ml/min on a 4.6 mm column, 0.85 ml/min on a 3.0 or 0.42 ml/min on 2.1 mm column.**

Helpful References

1. **Agilent 1200 Series Diode Array and Multiple Wavelength Detector SL User Manual. G1315-90011, February 2006.**
2. **Maintaining Your Agilent LC and LC/MS Systems, 5990-4957EN, November 2009.**
3. **"*The Influence of Sub-Two Micron Particles on HPLC Performance*" 5988-9251EN.**
4. ***"Step-by-step upgrade of Agilent 1100 Series LC systems to Agilent 1200 Series Rapid Resolution LC systems for higher performance", Part 1. 5989-6336EN.***
5. ***"Step-by-step upgrade of Agilent 1100 Series LC systems to Agilent 1200 Series Rapid Resolution LC systems for higher performance", Part 2. 5989-6337EN.***
6. **"*Optimize Data Sampling Rate to Take Advantage of RRHT Columns",* 5989-5810EN.**