The Importance of a Perfect Connection in Capillary HPLC

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Abstract

In small-bore HPLC, especially in capillary column dimensions, the quality of the separation can be greatly reduced by factors outside of the analytical column. While the influence of the end-user on fundamental pump and detector design may be limited, the choice of connection tubing and the way connections are made by the chromatographer can have a very significant impact on data quality.

The objective of capillary LC is the quantification of very small sample amounts, such as a low abundance impurity in a pharmaceutical product or a trace amount of protein or peptide in a biological sample.

Both adsorption of sample components on tubing walls or other materials and excessive band-spreading or mixing in open flow channels can lead to significant problems in capillary dimensions. This paper will focus on the latter issue.

Small-Bore LC Column Evolution

- It has been 100 years since Tswett published his pioneering LC work on plant pigment separation by what he called “column chromatography”.
- Main small-bore column advantages of greater mass sensitivity and easier interface to certain detectors such as mass spectrometry were documented in the early 1980s by Scott (1) and others.
- Practical applications of small-bore columns, especially below 2mm ID, have been rather slow to develop due to the very limited availability of optimized LC instruments, columns and accessories.

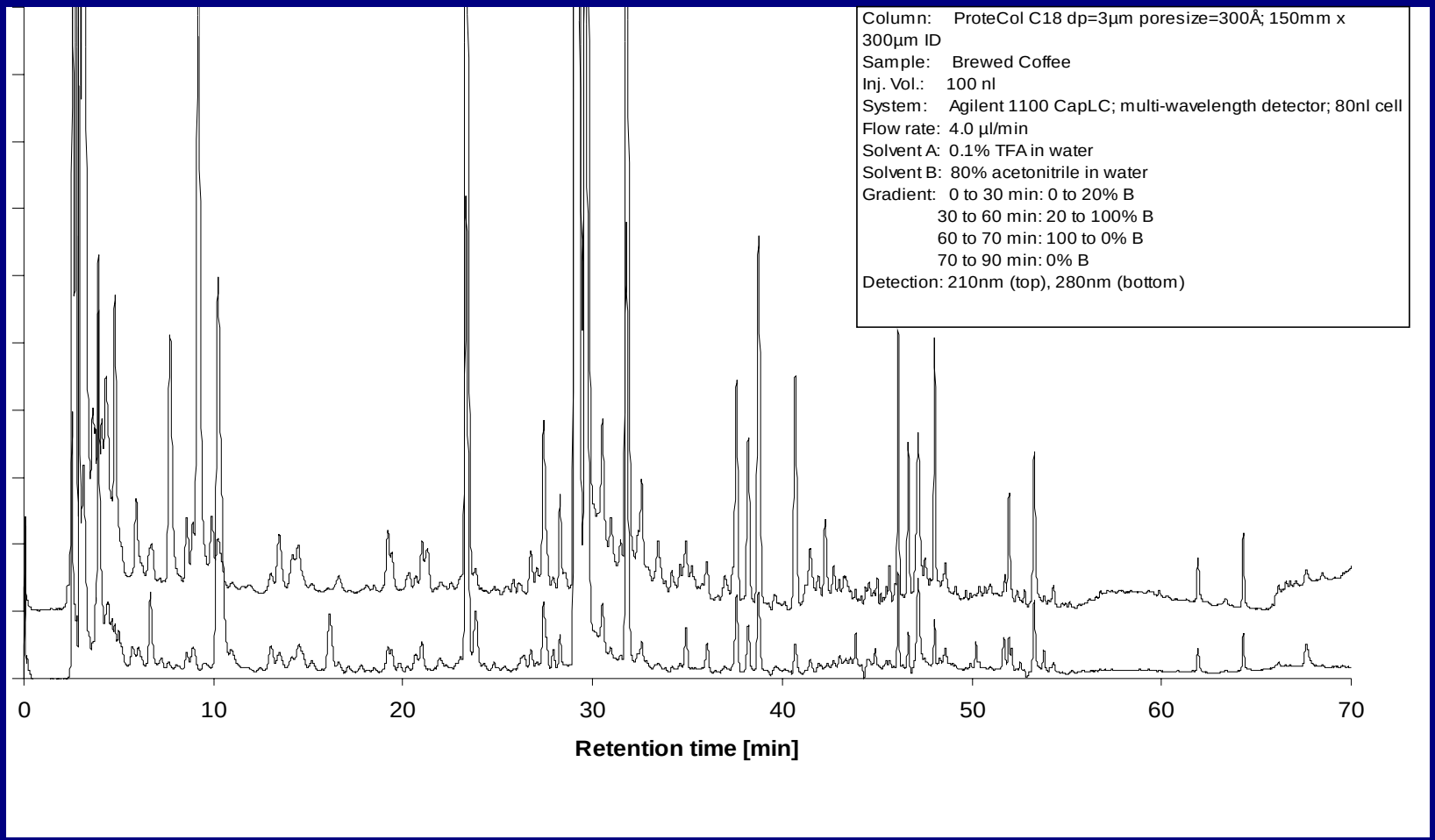
Some Operating Characteristics of Small-Bore Columns

ID (mm)	Typical Flow Rate (mL/min)	Typical Flow Rate (uL/min)	Mass Sensitivity	Solvent Consumption
4.6	1.000000	1000	1	1
4	0.756144	760	1.3	0.76
2	0.189036	200	5.3	0.2
1	0.047259	47	21	0.047
0.5	0.011815	12	85	0.012
0.3	0.004253	4.2	235	0.0042
0.15	0.001063	1.1	940	0.0011
0.075	0.000266	0.27	3762	0.00027

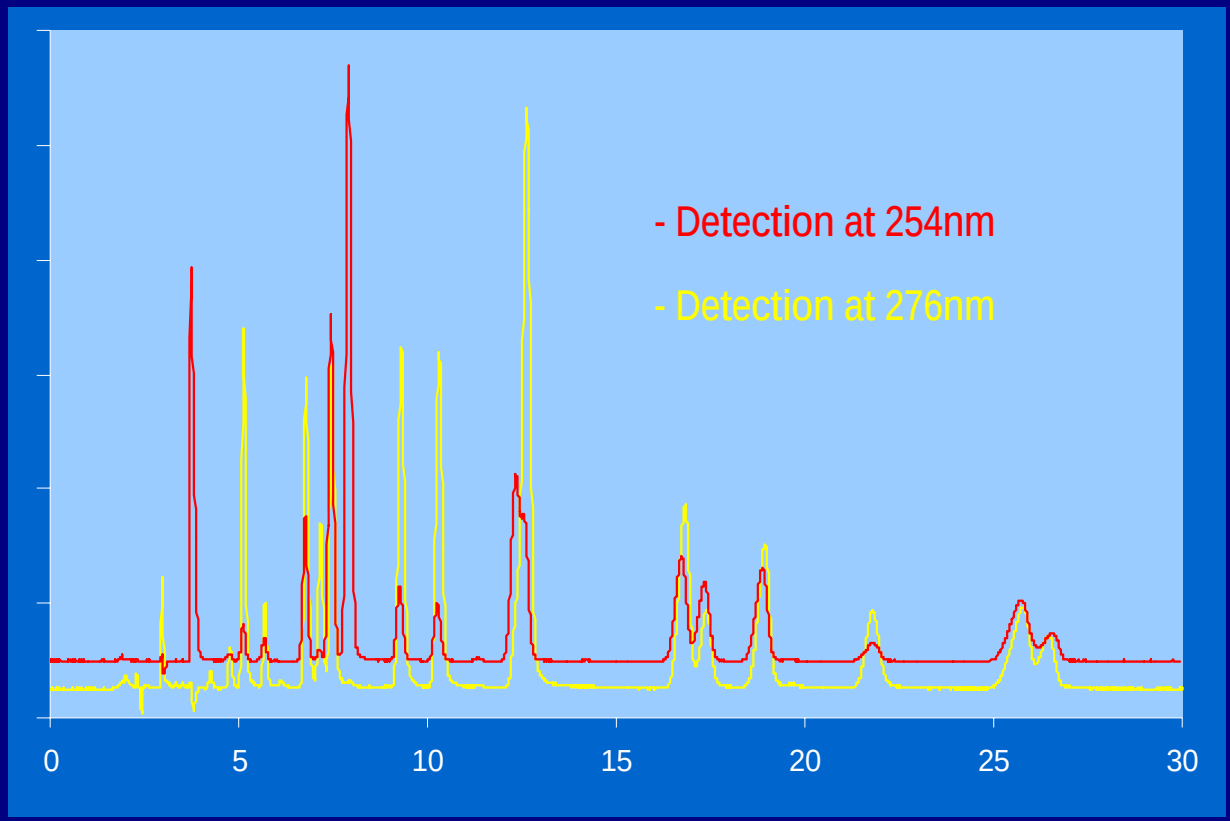
It is a misconception that small-bore LC columns yield greater efficiency than larger ID columns of the same length; more plates can only be realized when small-bore columns of greater length can be made and other components can be optimized.

Brewed Coffee

ProteCol C18, 3um, 300A



PAHs on 300µm ID ProteCol™ LC Capillary Column



Column: 300µm x 150mm
ProteCol C18, 3µm, 120Å
80% AcCN : 20% water
4.0 µl/min

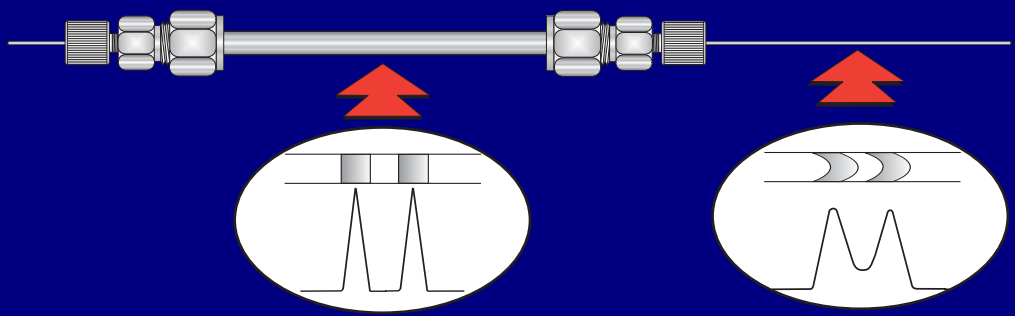
16 polyaromatic hydrocarbons (EPA 610)

50:1 flow split after injector.

Peak volumes defined by 4**s** can be less than 1 **µL** in capillary LC columns; therefore, volumes of other components must be much smaller than 1 µL

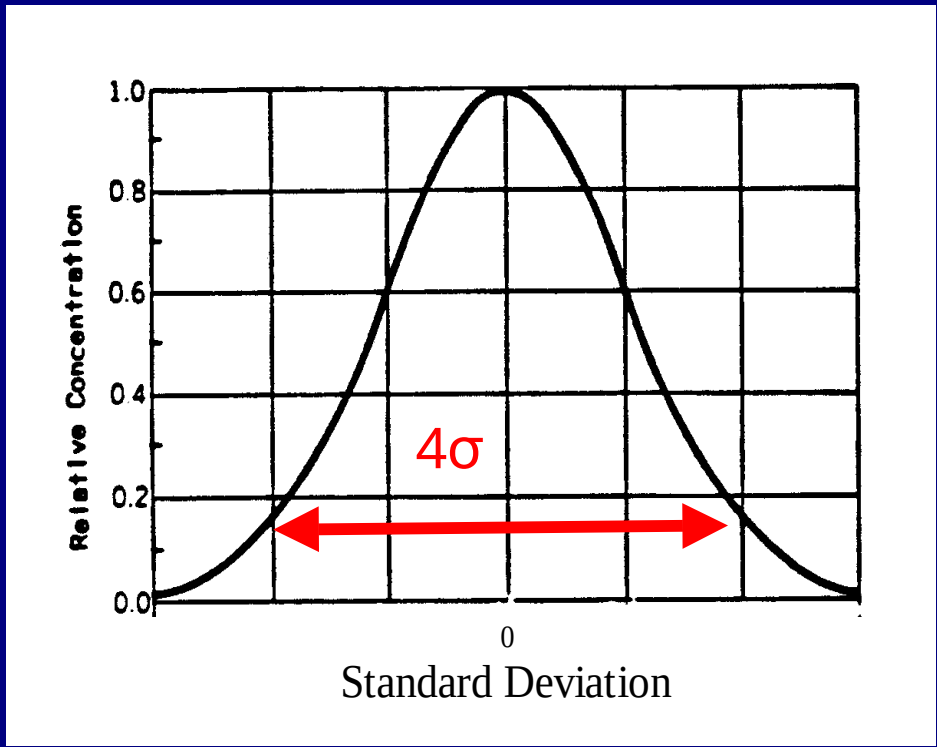
Peak or Band Dispersion

- Peaks typically show Gaussian or random distribution defined by the standard deviation, s , expressed in time, volume or distance units.
- Dilution or dispersion by mobile phase occurs as solutes pass through the chromatographic system.
- Dispersion can occur both inside and outside of the column bed. Separation gained in the column can be lost in connectors, especially after the column.



Gaussian Peak Shape

$\sigma_{col} = V_0 (1 + k) / N^{1/2}$ (dispersion in a packed column bed)



Peak volume is often estimated as 4s at 13.4 % of peak height. It is proportional to column bed volume, V_0.

Peak volume is determined by the effects of the column bed (equation) plus the effects of other system components.

Broad distribution destroys resolution and sensitivity.

Dispersion in Column Bed[2,3]

$$\sigma^2 = V_o^2(1 + k')^2/N$$

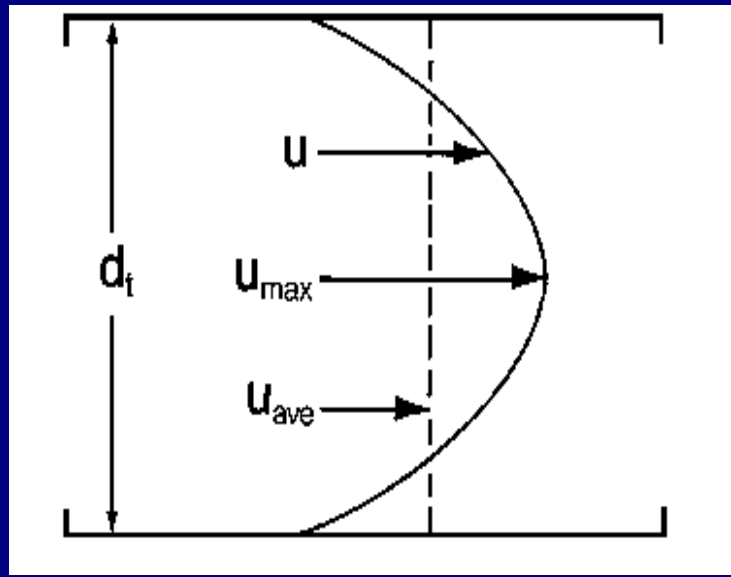
where variance has units of μL^2 when

V_o = mobile phase volume of column in µL
(unretained peak retention volume)
k' = capacity factor of the peak
N = number of theoretical plates of column

Small geometry, short retention and high efficiency favor low dispersion in columns.

Dispersion in Open Connectors[2,3]

$$\sigma^2 = 1.36 \times 10^{-3} d_t^4 L_t F/D$$



Velocity at the wall is essentially zero under laminar flow conditions. Small inside diameter, short length, low flow and fast diffusion favor low dispersion in connection tubes and accessories. Larger molecules show greater dispersion (1/D).

Total Peak (Band) Dispersion Expressed as Variance Equation[3,4]

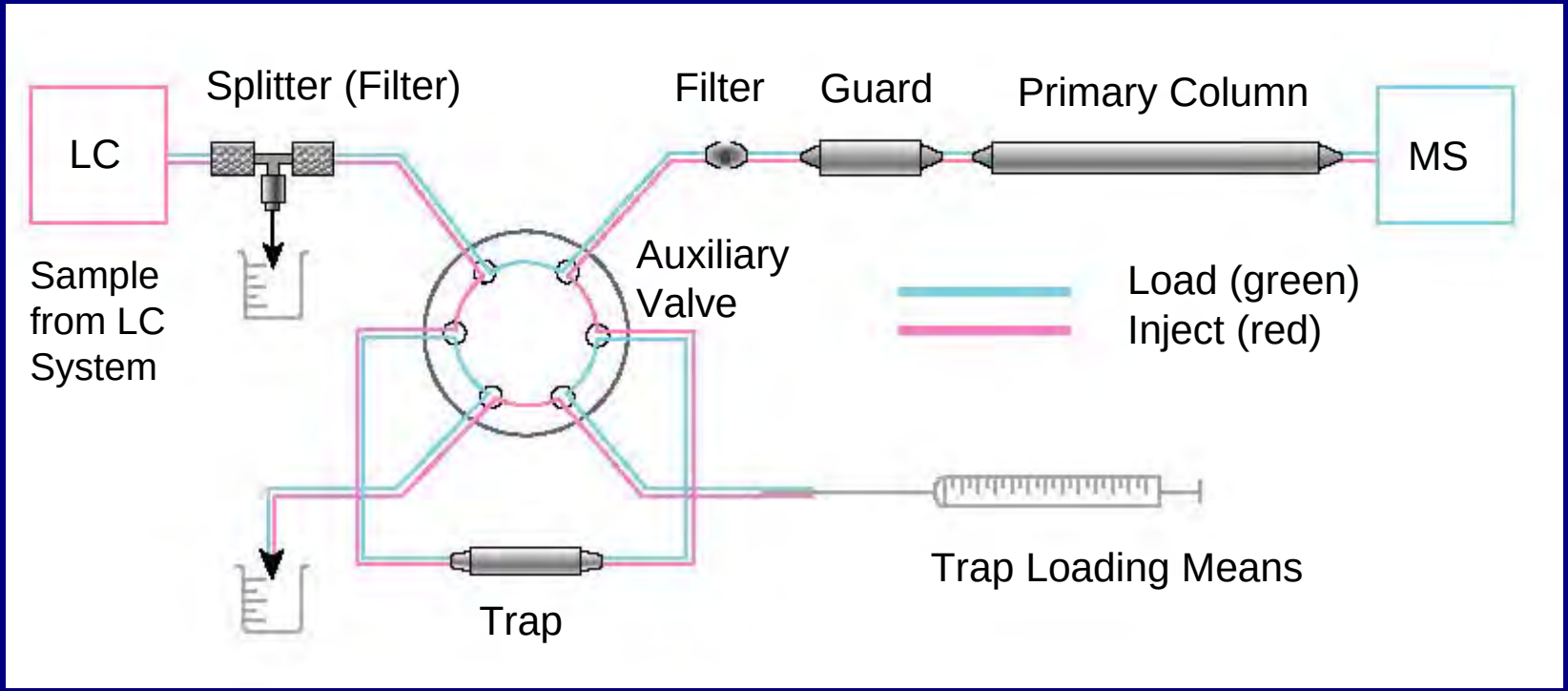
$$\sigma^2_{tot} = \sigma^2_{col} + \sigma^2_{inj} + \sigma^2_{det} + \sigma^2_{conn}$$

System Constant (σ_{sys})

- Component design determines the System Constant; they often can be optimized by the user for different column situations.
- Sometimes an additional variance term is added to account for thermal mismatch of solvent temperature as sample passes from ambient into a heated or cooled column bed.

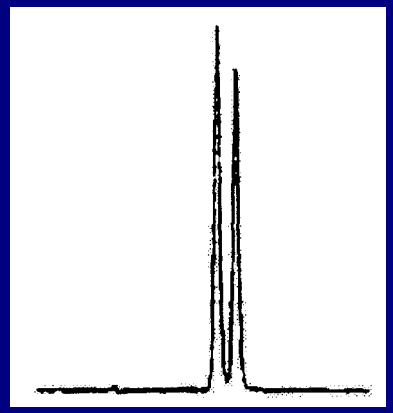
Example of a Capillary LC Flow Path

Small-volume components are critical to success

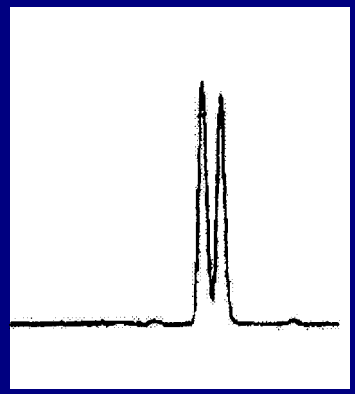


Effect of Tubing Dispersion on 100x2mm Column

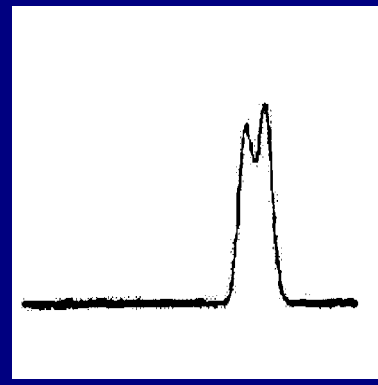
.010 inch I.D.



5-cm long

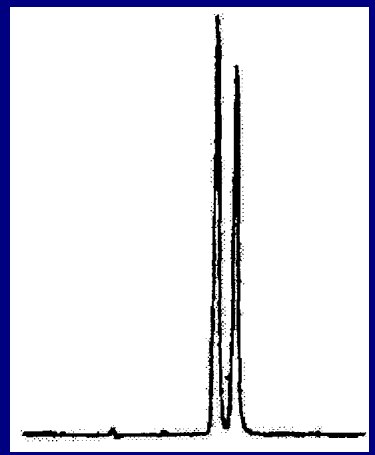


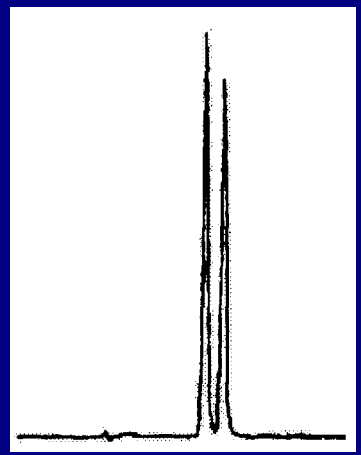
20-cm long

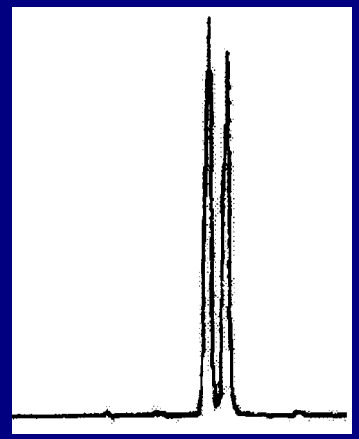


80-cm long

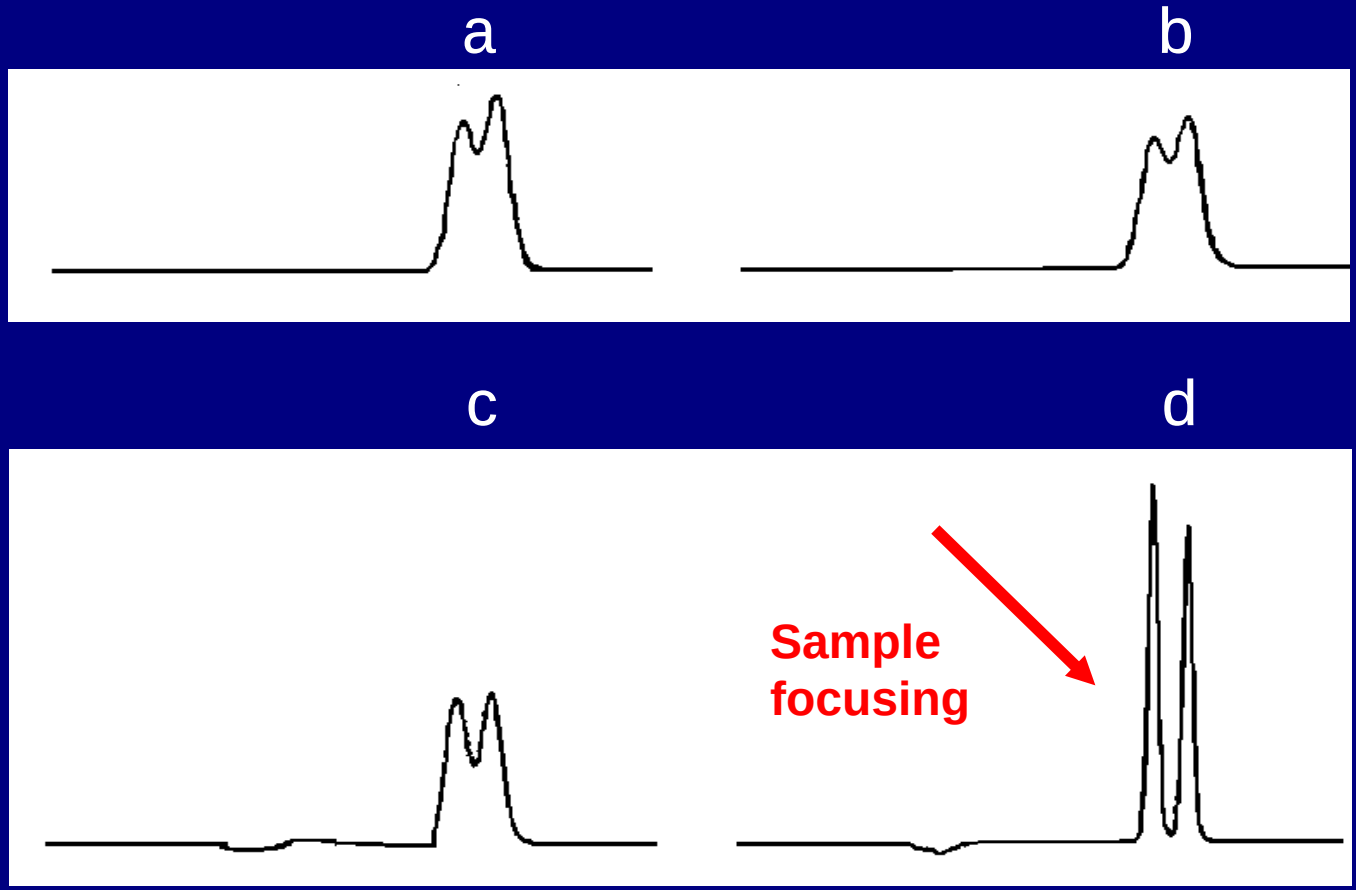
.005 inch I.D.







Effect of Tubing Location on Dispersion[3]



Tubing after the column always disperses the sample

Gradient Elution Advantages

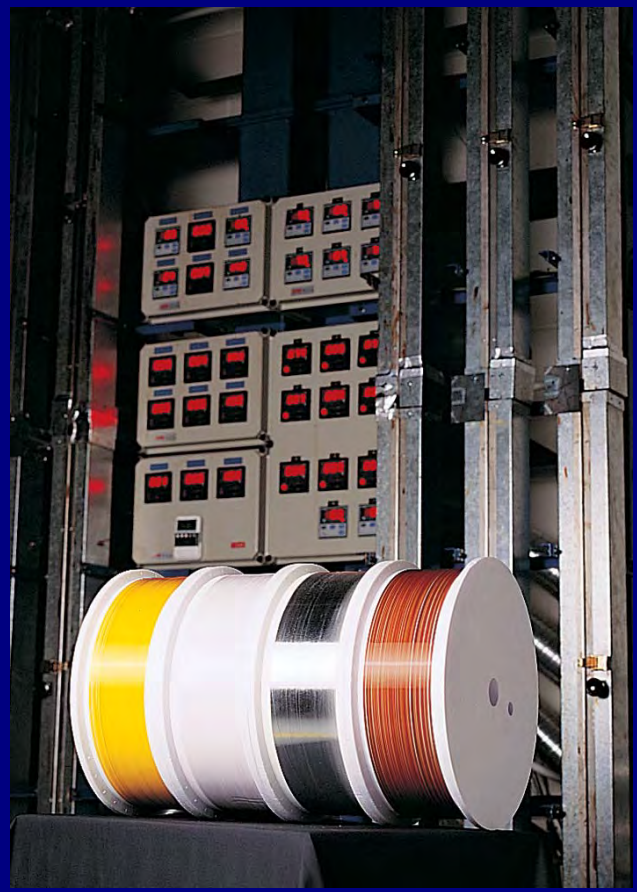
- Isocratic elution creates increasing dispersion as a function of efficiency (N), void volume (V_0) and retention (k), causing lower sensitivity for more retained solutes.
- Gradient elution focuses sample components at the column inlet and creates uniform dispersion for all solutes by reducing or eliminating the retention factor aspect.
- In the past, gradients have not been practical for trace analysis due to solvent-related detector noise and drift.
- MS with volatile mobile phases does not have this solvent limitation, so gradient experiments can be conducted at high sensitivity if reliable solvent programs can be generated at low Capillary LC flow rates.

Optimizing Capillary LC Components

The ProteCol™ System

- Integrated approach to Capillary LC incorporating columns, unions, tubing, splitters, filters and accessories.
- All components perfectly matched for minimum dispersion volume.
- Featuring PEEKsil™ fused silica lined PEEK tubing.
- Unique, convenient designs virtually eliminate the dispersive effects of components having drilled holes.
- Create a complete column system or improve a system with ProteCol components.

Fused Silica Shows Advantages



One of four drawing towers located at SGE Melbourne, AU

Fused Silica Lined Tubing Benefits[5]

PEEK Tubing:

- Often produces a rough-cut
- Potential dead volume
- Structural integrity greatly affected by solvent, temperature and pressure

PEEKsil Tubing:

- Square cut and polished ends
- No dead volume
- Silica liner protects structural integrity of polymer sheath

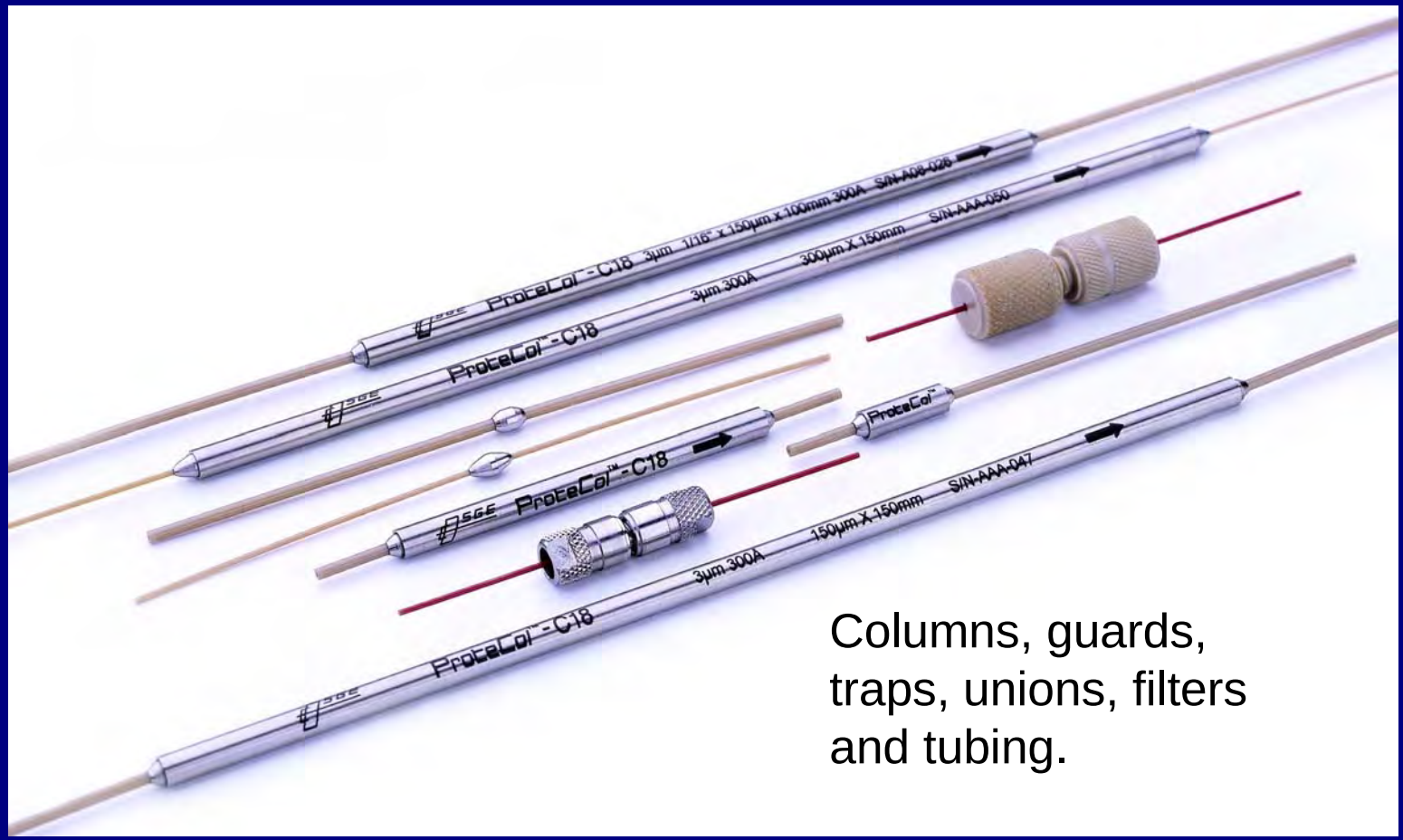
A microscope or good magnifying glass is an important tool for inspecting connectors in Capillary LC.

Volume Contribution of Standard PEEKsil™ Connection Tubing Lengths

Internal Tubing Volume in µL						
		Tubing length [cm]				
Tubing ID [µm]		5	10	15	25	50
	25	0.00	0.00	0.01	0.01	0.02
	50	0.01	0.02	0.03	0.05	0.10
	75	0.02	0.04	0.07	0.11	0.22
	100	0.04	0.08	0.12	0.20	0.39
	150	0.09	0.18	0.27	0.44	0.88
	175	0.12	0.24	0.36	0.60	1.20
	200	0.16	0.31	0.47	0.79	1.57
	300	0.35	0.71	1.06	1.77	3.53

← 0.001” ID
← 0.002” ID
← 0.003” ID
← 0.005” ID
← 0.010” ID

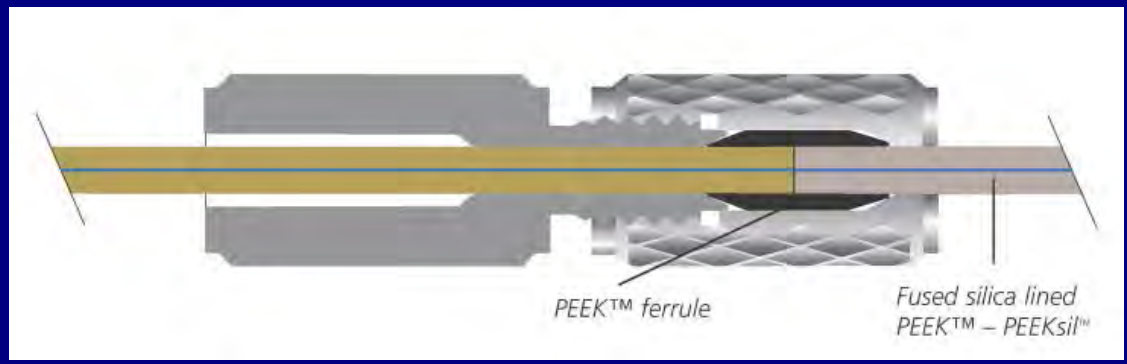
ProteCol™ Components

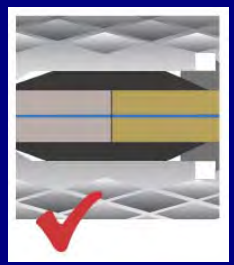


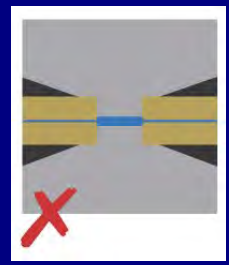
Columns, guards, traps, unions, filters and tubing.

ProteCol™ Components Employ a Unique Ferrule Concept

- Keeps system dead volume and total volume to an absolute minimum.
- Allows tubing or column connections without adding volume from the fitting body.

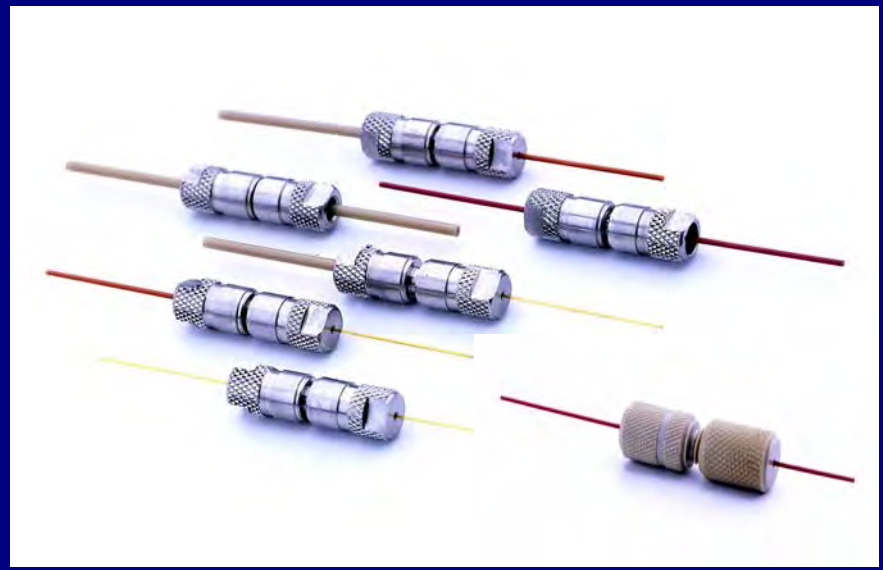






ProteCol™ Unions

- Butt tubing connections avoid dead volume and extra volume.
- Each ProteCol Union has a different ferrule.
- Flats for wrench tightening at higher pressures.
- Knurls allow finger tightening at lower pressures.

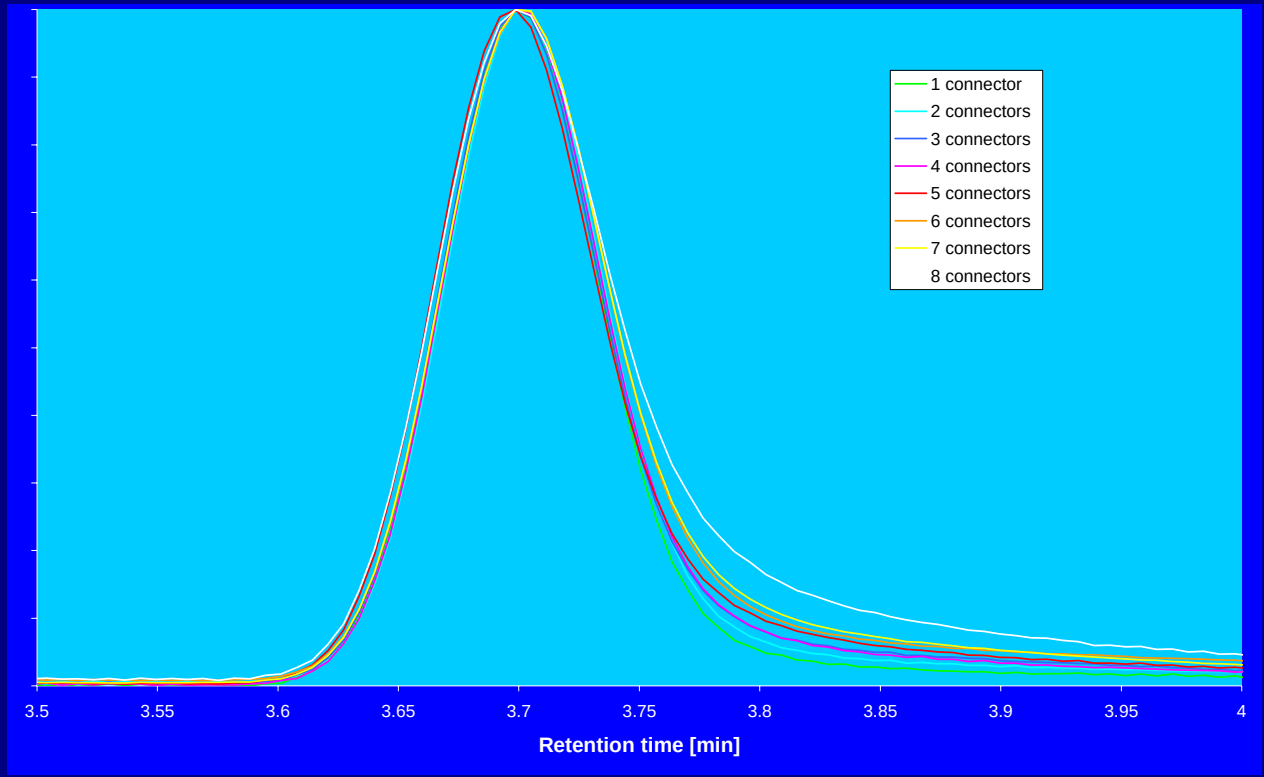


Industry-wide dimension standards are not yet in place for tubing and fittings. SGE tubing, fittings and ferrules are closely controlled for tight fit and reliable high pressure use.

Influence of Multiple 50 µm ID Connections on Peak Shape

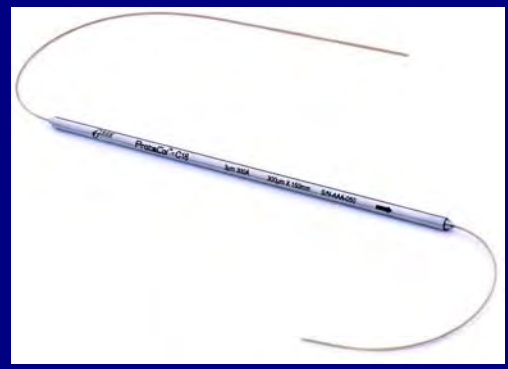
ProteCol C18 100mm x 300µm Column extended with a total of 40cm PEEKsil tubing (50µm ID) incorporating 1 to 8 zero volume connections.

Flow rate: 4.0 µl/min
Mobile phase: 60% ACN in water
Naphthalene peak shown



ProteCol™ Capillary Analytical Column

- No endfittings required.
- Efficient butt connections
- Rigid 1/16” and flexible 1/32” versions available.



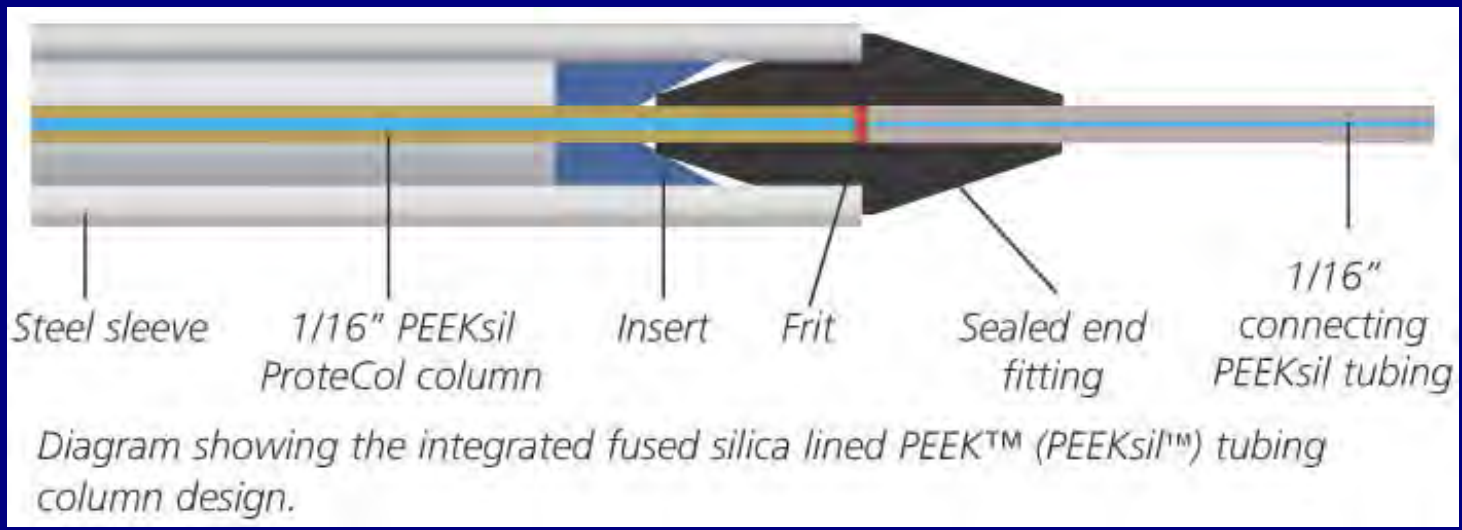
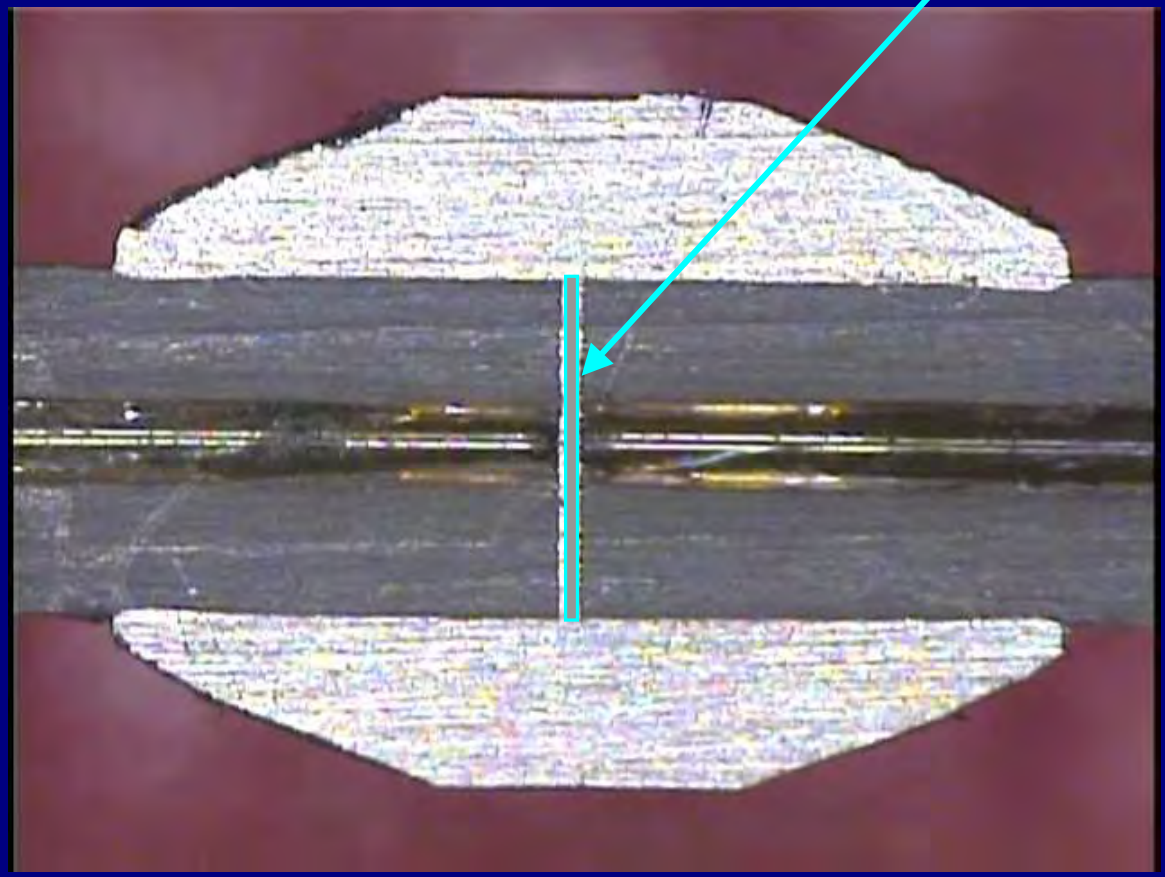
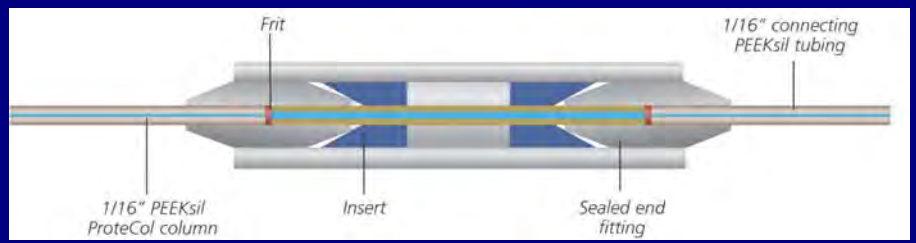


Diagram showing the integrated fused silica lined PEEK™ (PEEKsil™) tubing column design.

ProteCol™ Column Endfitting[6]

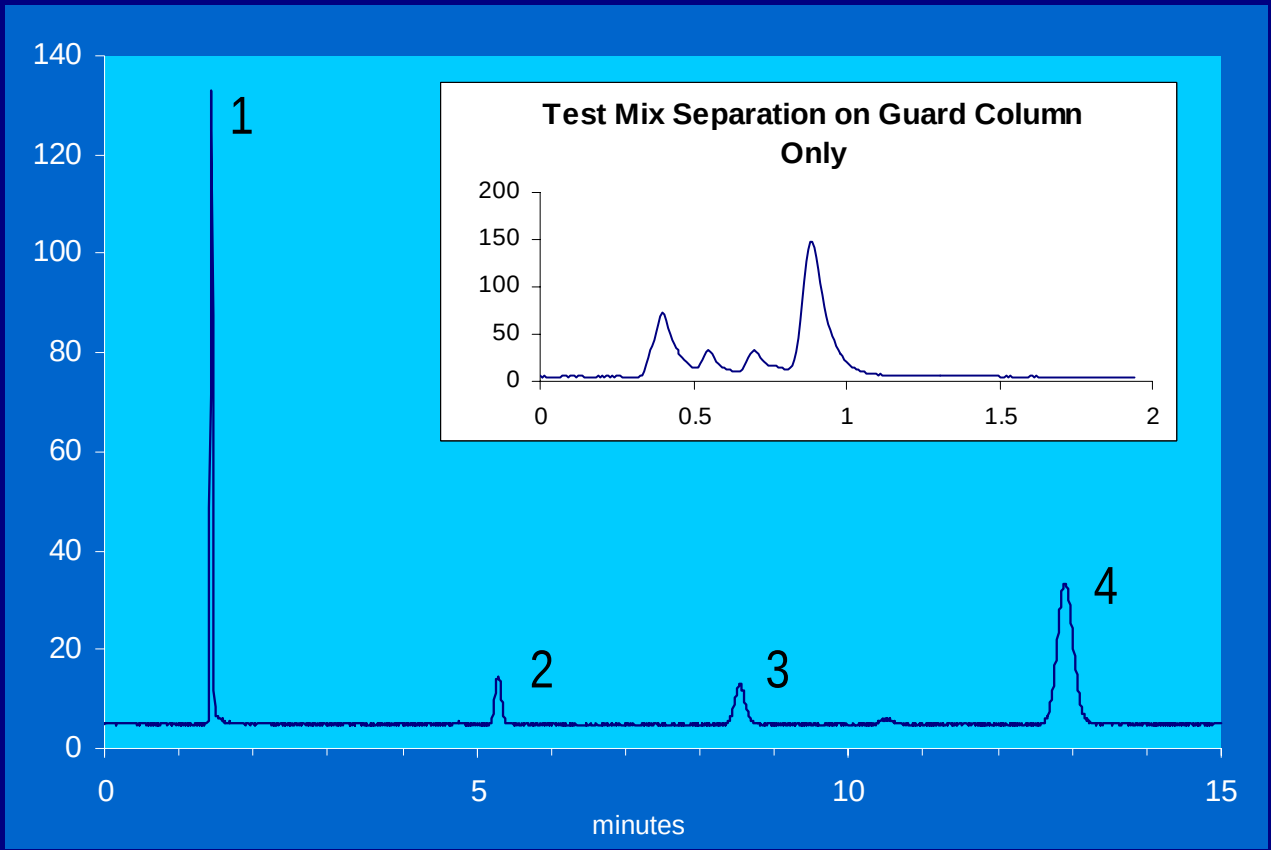


ProteCol™ Capillary Guard Column



- Bed is 1 cm length.

Test Chromatograms on ProteCol™ Capillary Columns



Column: 300µm x 150mm
ProteCol C18, 3µm, 120Å
60% AcCN : 40% water
4.0 µl/min

1 Pyridine
2 Methyl Benzoate
3 Phenetole
4 Naphthalene

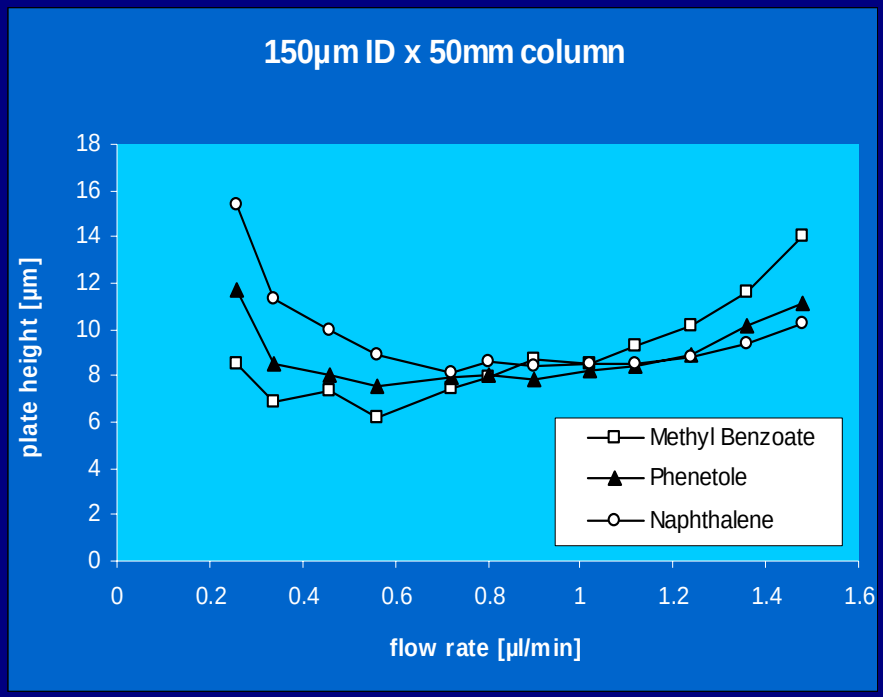
50:1 flow split between injector and column.

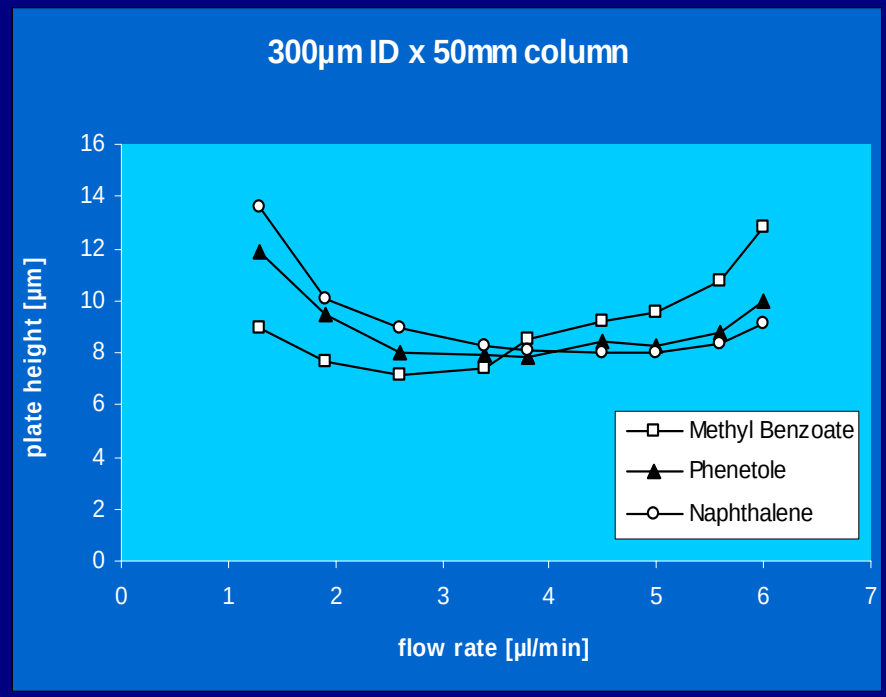
Current Phases for the ProteCol™ Column Range

Phase	dp	ligand	pore size	carbon load	surface area	pore volume
ProteCol C18-120-3	3 µm	C18 endcapped	120 Å	17%	350m²/g	1.1ml/g
ProteCol C18-120-5	5 µm	C18 endcapped	120 Å	17%	350m²/g	1.1ml/g
ProteCol C18-300-3	3 µm	C18 endcapped	300 Å	9%	100m²/g	0.9ml/g
ProteCol C18-300-5	5 µm	C18 endcapped	300 Å	9%	100m²/g	0.9ml/g
ProteCol C4-300-3	3 µm	C4	300 Å	3%	100m²/g	0.9ml/g
ProteCol NH2-300-3	3 µm	-NH2	300 Å	2%	100m²/g	0.9ml/g

- ProteCol C18-120 phases are optimized for separation of small molecules and are highly deactivated.
- ProteCol C18-300 phases are optimized for separation of biomolecules such as peptides and proteins.

Van Deemter Plots for ProteCol™ LC Capillary Columns





- **ProteCol C18-120-3 (3µm; 120Å)**

ProteCol™ Capillary Trap Column

- Allows preconcentration of a sample before injection onto the analytical column.
- Short, flexible design allows easy, efficient valve installation.
- 10mm length standard

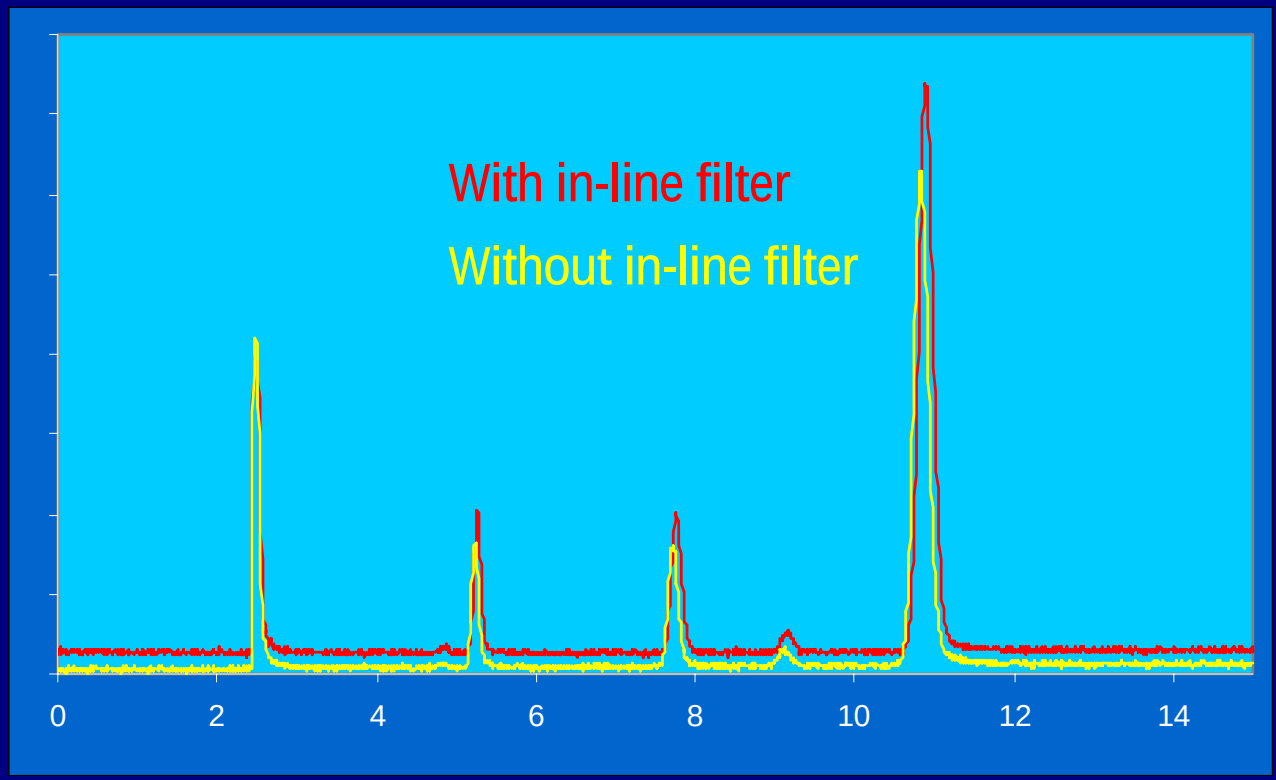
ProteCol™ Capillary LC Filtering Connector

1/32” OD

1/16” OD

- Two filter versions protect Capillary LC columns (2um and 50 micron tubing is standard)

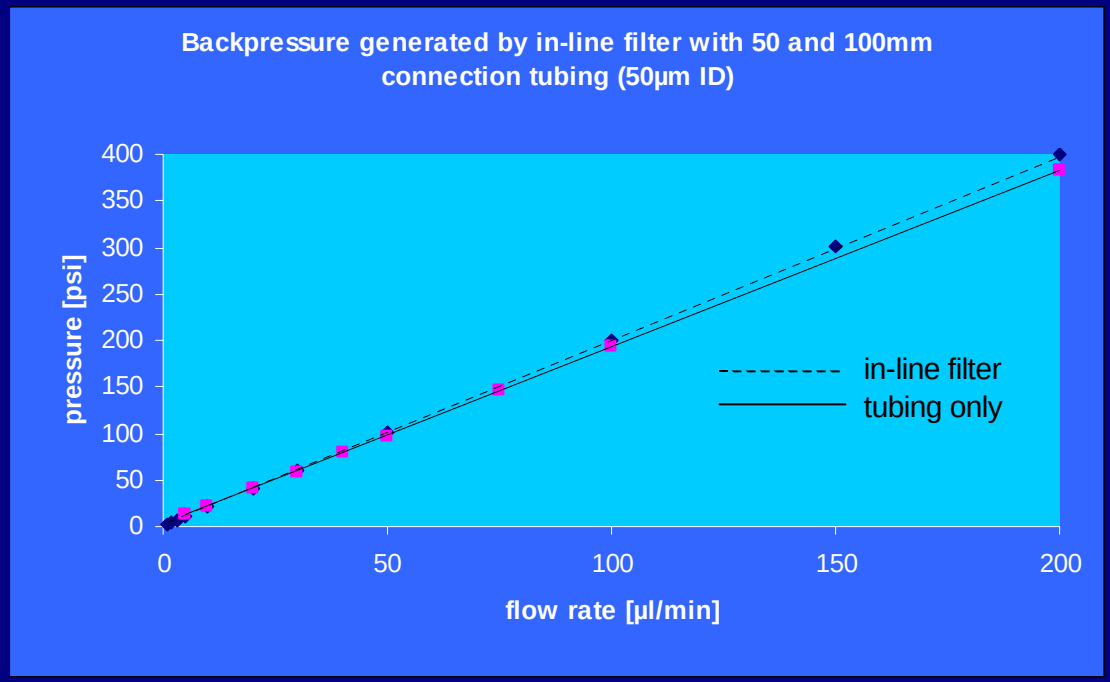
Affect of Filtering Connector on Column Performance



Column: 300µm x 150mm
ProteCol C18, 3µm, 120Å
60% AcCN : 40% water
4.2 µl/min

Test Mix:
Pyridine
Methyl Benzoate
Phenetole
Naphthalene

Back Pressure Contribution of the Capillary Filtering Connector



The pressure drop is entirely across the tubing, not the filter element. A larger ID Micro version of the filtering connector is available for 1 and 2mm ID columns.

The 2µm porosity filter connected to 50µm ID tubing of 150mm total length gave a slope of only 2 psi per 1 µL/min flow, which should be ideal for columns of 1mm ID or smaller.

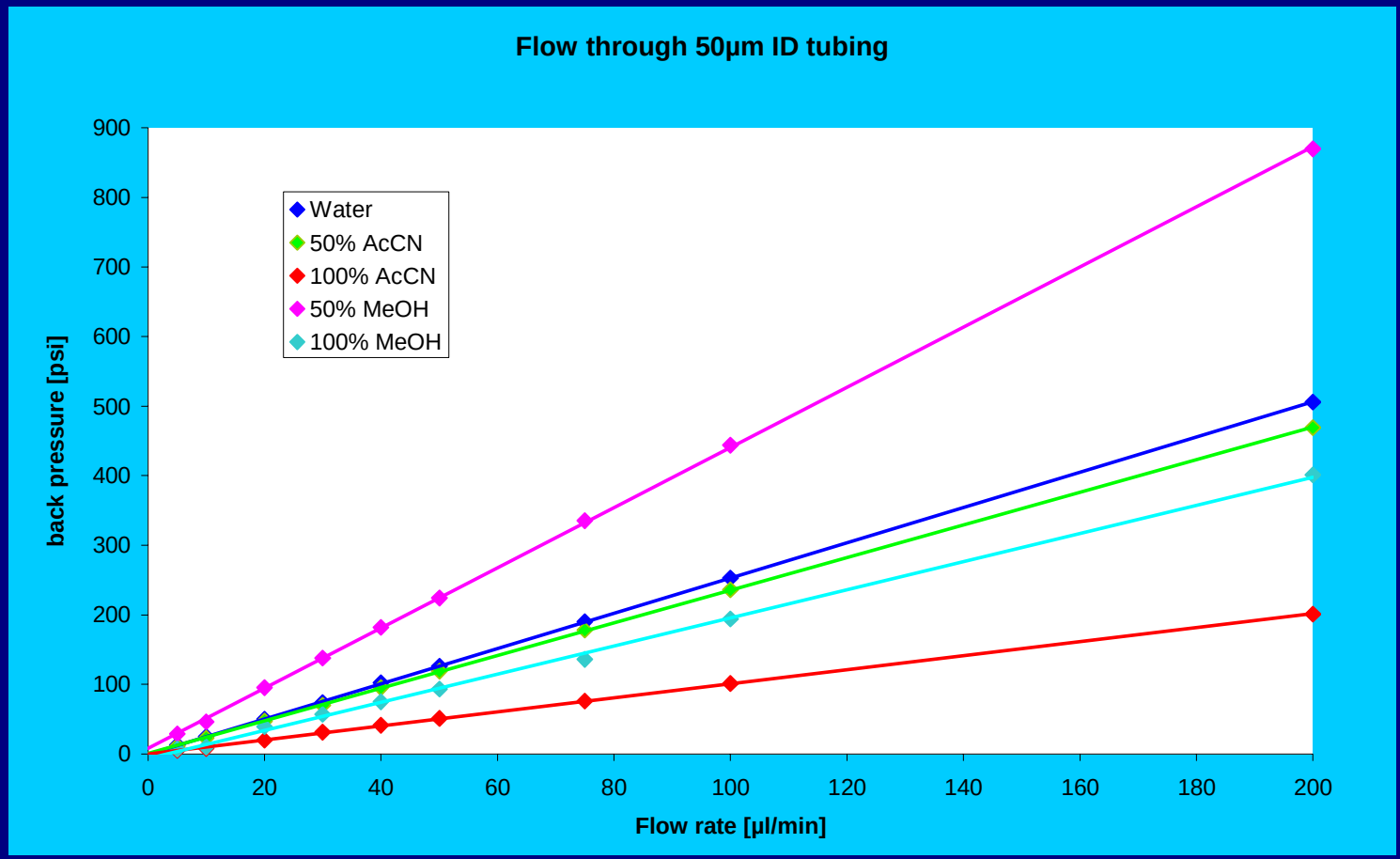
Backpressure in Capillaries

The backpressure generated in a capillary is described by the Poiseuille's equation:

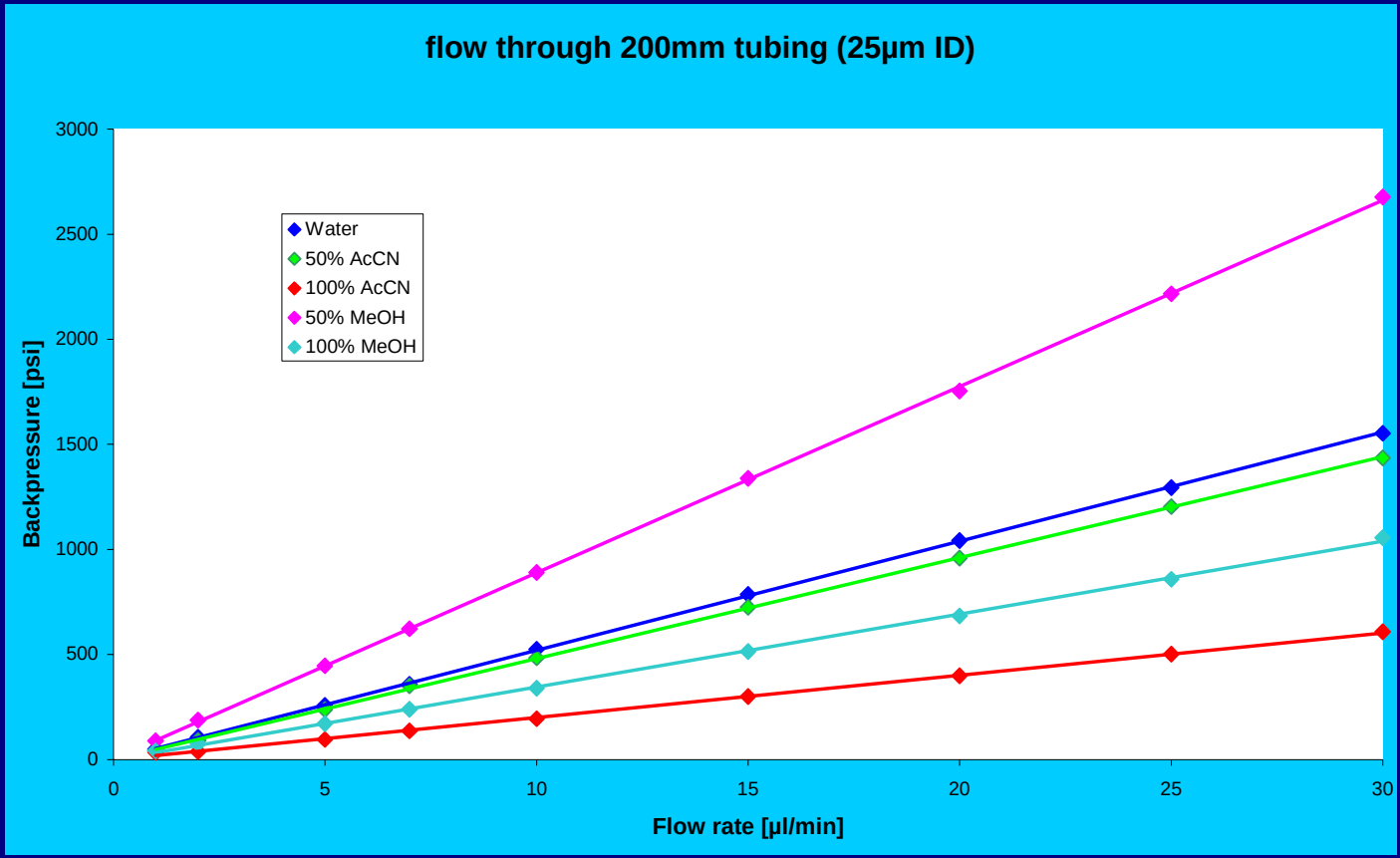
$$\Delta p = \frac{F \cdot 8\eta \cdot l}{\pi \cdot r^4}$$

Δp = pressure drop
η = viscosity
F = flow rate
r = capillary radius
l = length

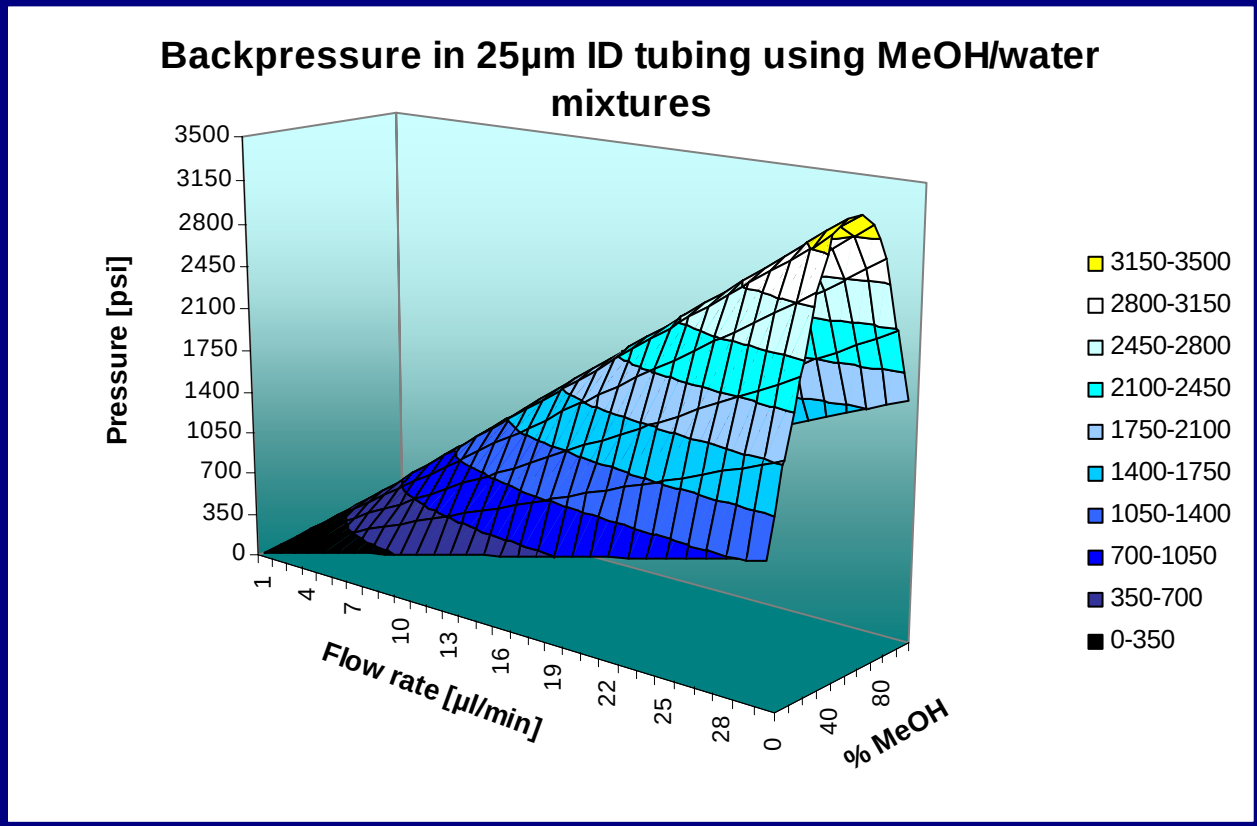
Measured Pressures in 50 µm ID Tubing



Measured Pressures in 25 µm ID Tubing

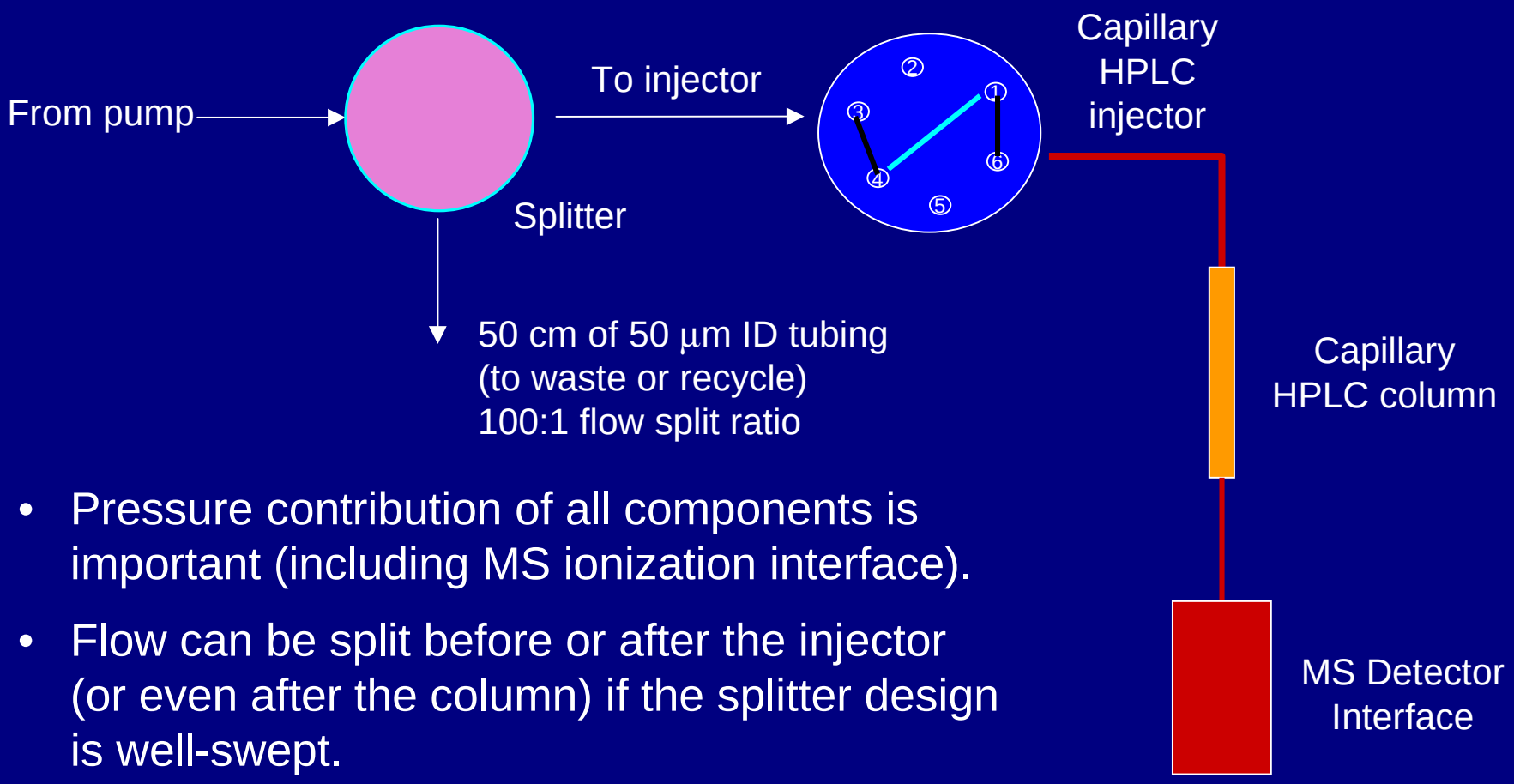


Influence of Mobile Phase on System Pressures



A mixture of 60% MeOH in water has a much higher viscosity than either MeOH or water alone.

A Typical Split Flow System



- Pressure contribution of all components is important (including MS ionization interface).
- Flow can be split before or after the injector (or even after the column) if the splitter design is well-swept.

ProteCol™ Low Dispersion Splitter

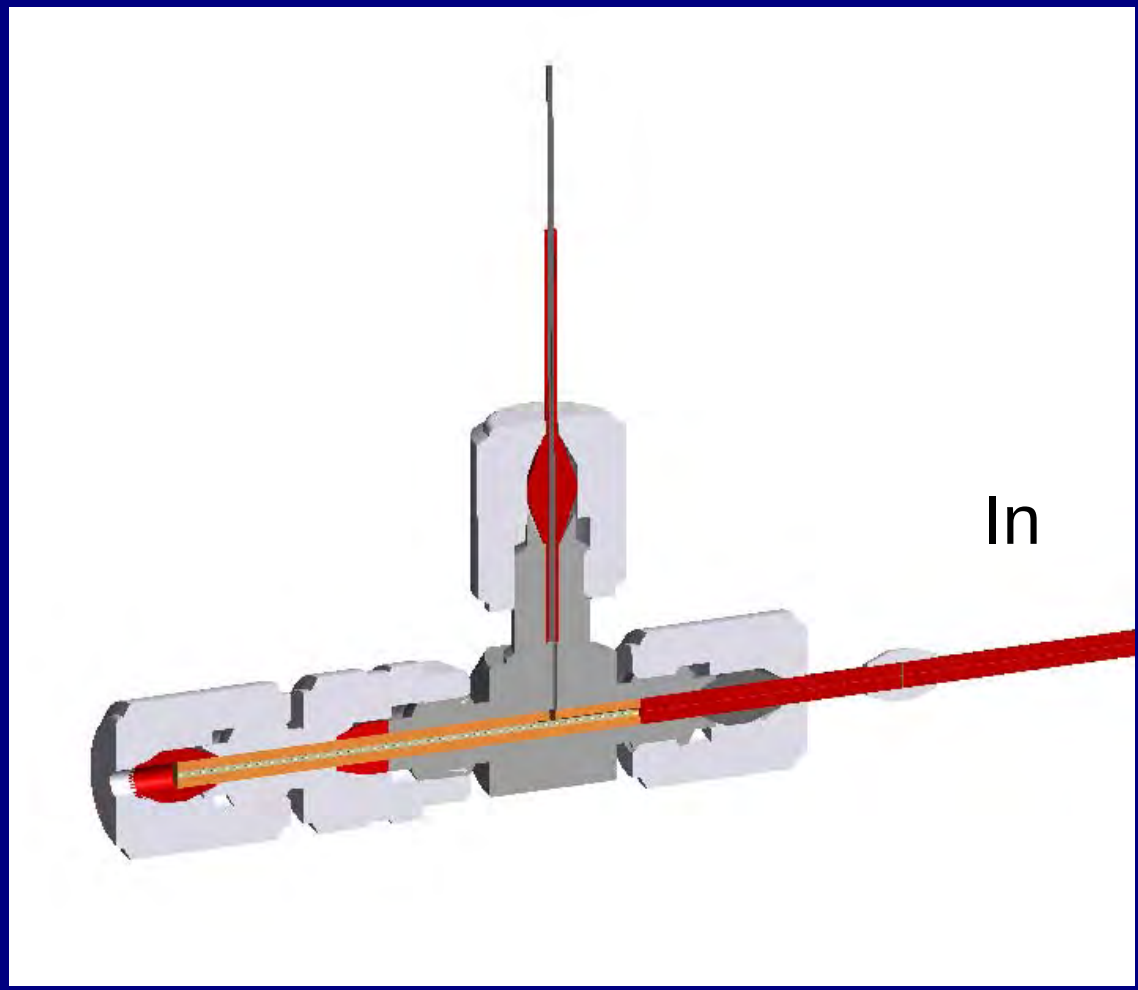
Control the sample flow to the column by changing the length of the bypass tube.

Can be used:

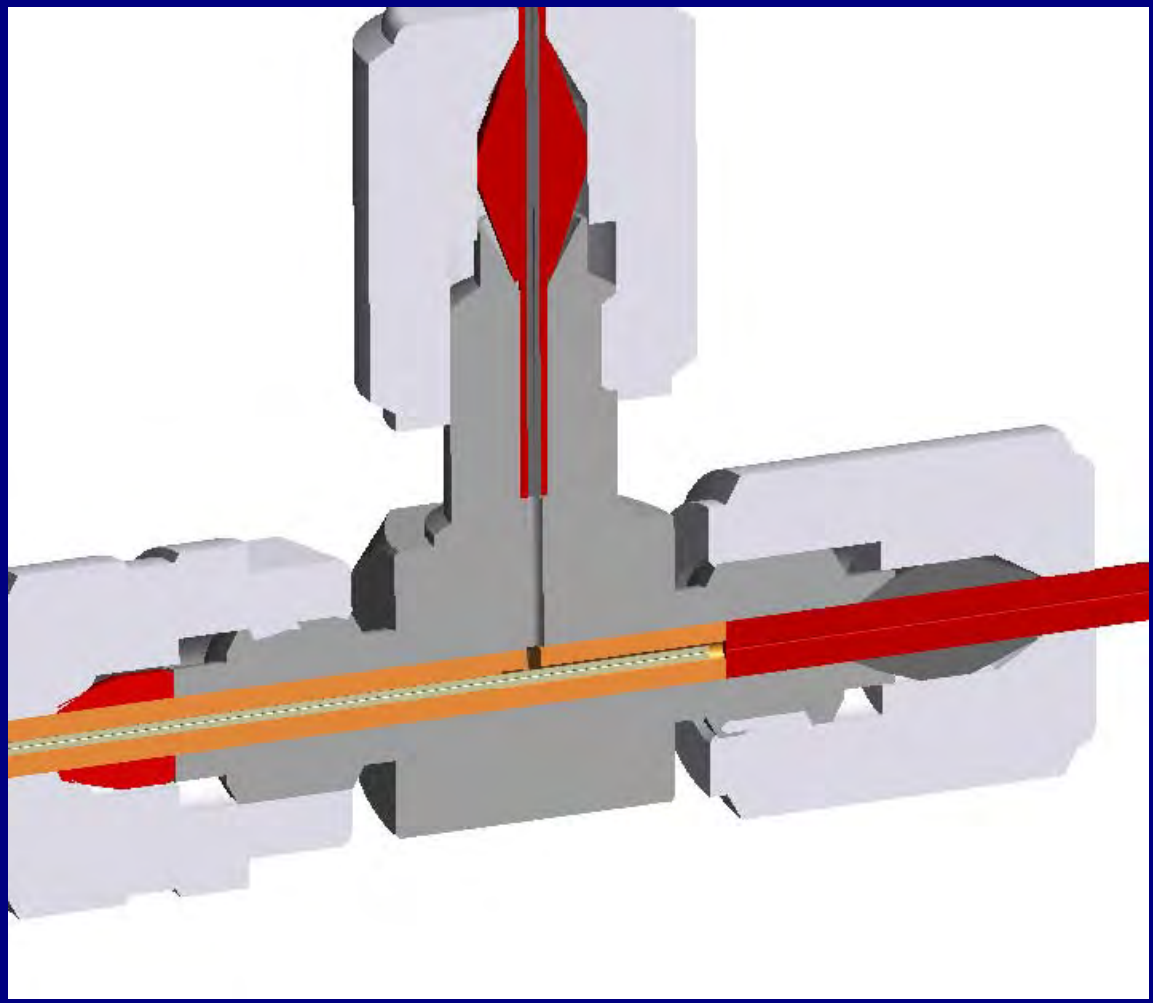
- Before the injector
- Between injector and column(s)
- Between column(s) and detector
- Extremely small internal dimensions and volume allow sample to pass through with minimal dispersion.

Splitter features a 2µm integral filter at the inlet.

Splitter Sectioned Drawing

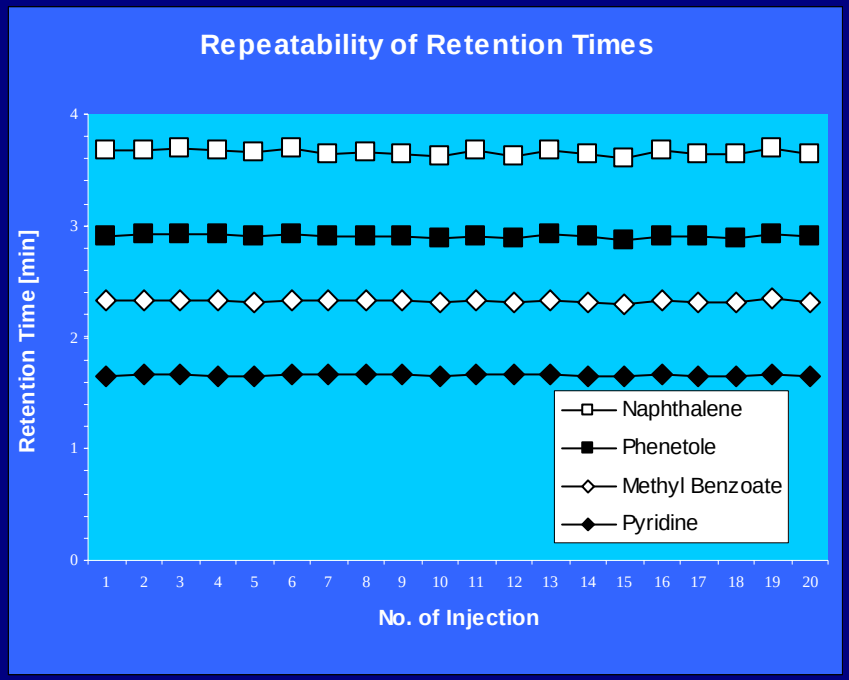


Splitter Section Close-up



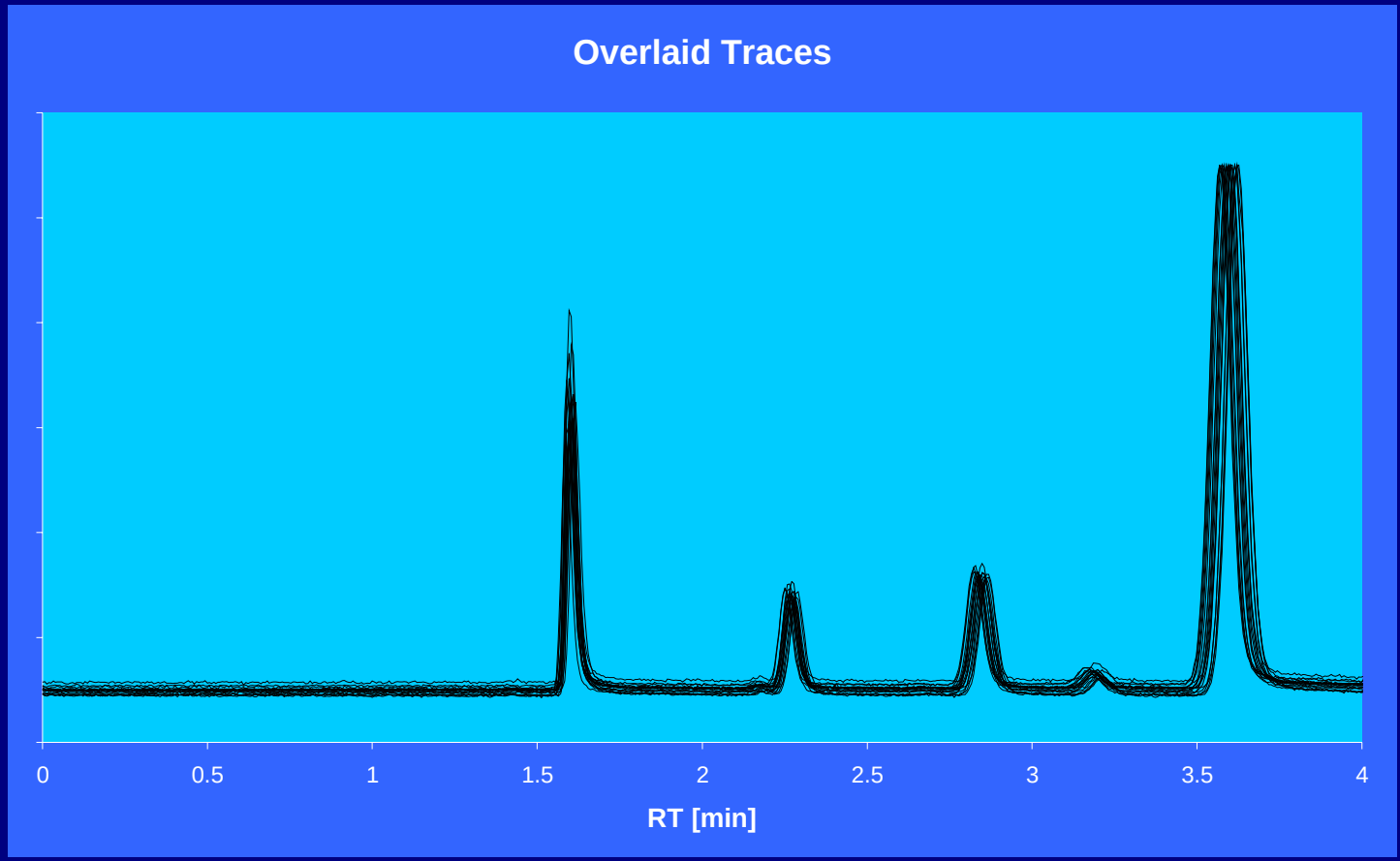
Repeatability of Column and Splitter Performance

	Pyridine	MetBenz	Phenetole	Naphthalene
1st inj.	1.65	2.34	2.91	3.67
2nd inj.	1.66	2.34	2.92	3.68
3rd inj.	1.67	2.34	2.93	3.69
4th inj.	1.65	2.33	2.92	3.68
5th inj.	1.65	2.32	2.90	3.66
6th inj.	1.67	2.34	2.93	3.69
7th inj.	1.66	2.33	2.90	3.64
8th inj.	1.66	2.33	2.91	3.66
9th inj.	1.66	2.33	2.90	3.64
10th inj.	1.65	2.31	2.89	3.63
11th inj.	1.67	2.33	2.91	3.67
12th inj.	1.66	2.32	2.89	3.63
13th inj.	1.67	2.34	2.92	3.67
14th inj.	1.65	2.32	2.90	3.65
15th inj.	1.65	2.30	2.87	3.61
16th inj.	1.66	2.34	2.91	3.67
17th inj.	1.65	2.32	2.91	3.65
18th inj.	1.65	2.31	2.89	3.64
19th inj.	1.67	2.35	2.93	3.69
20th inj.	1.65	2.32	2.90	3.65
average	1.66	2.33	2.91	3.66
StdDev	0.01	0.01	0.02	0.02
%RSD	**0.50**	**0.55**	**0.54**	**0.62**

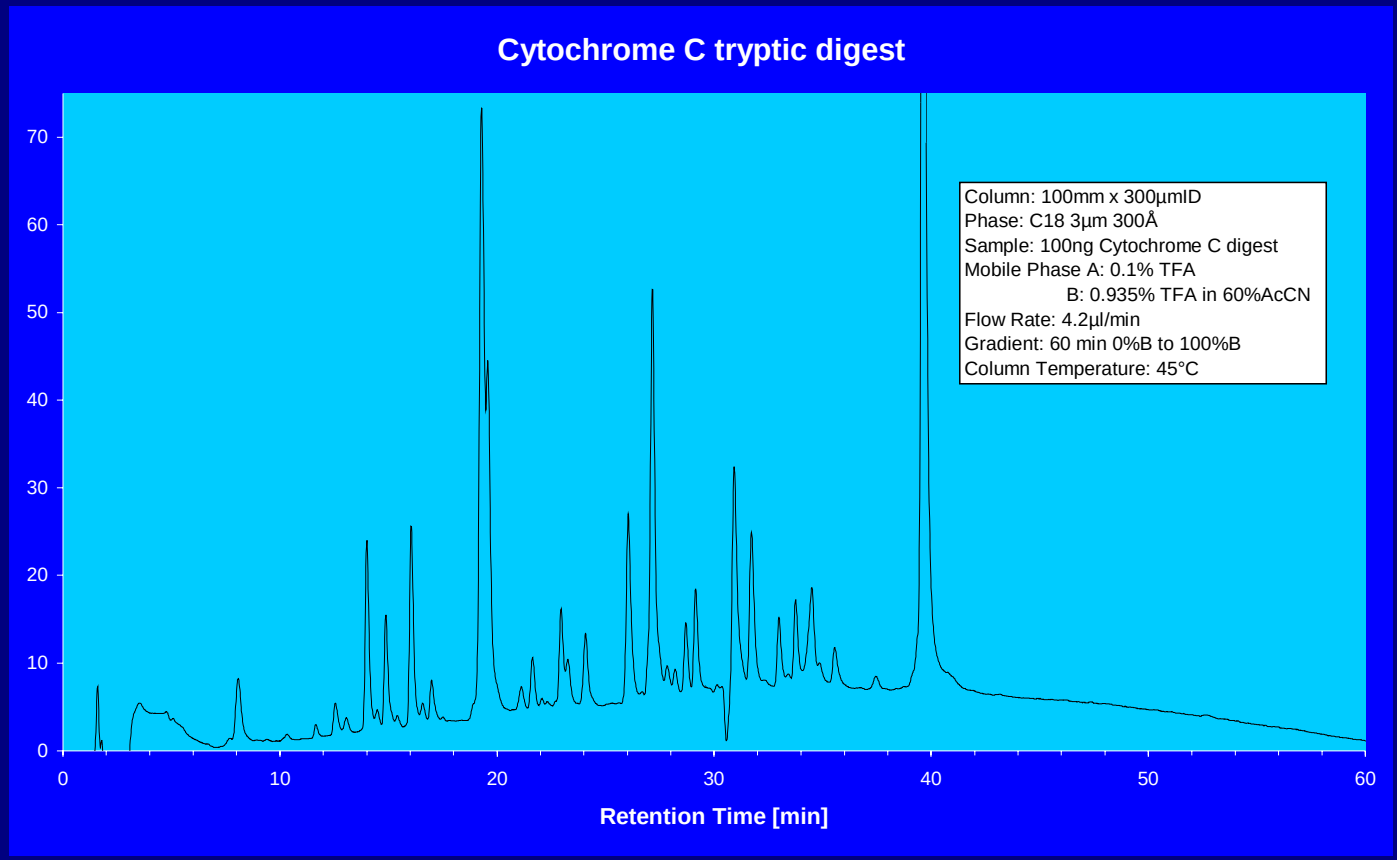


Column: 100mm x 300µm ProteCol C18, 3µm, 300Å
Mobile Phase: 60%AcCN:40% water
Flow Rate: 3.8µl/min
Wavelength: 254 nm
Flow splitting after injection valve

Overlaid Chromatograms from the Repeatability Experiment



Tryptic Digest of Cytochrome C



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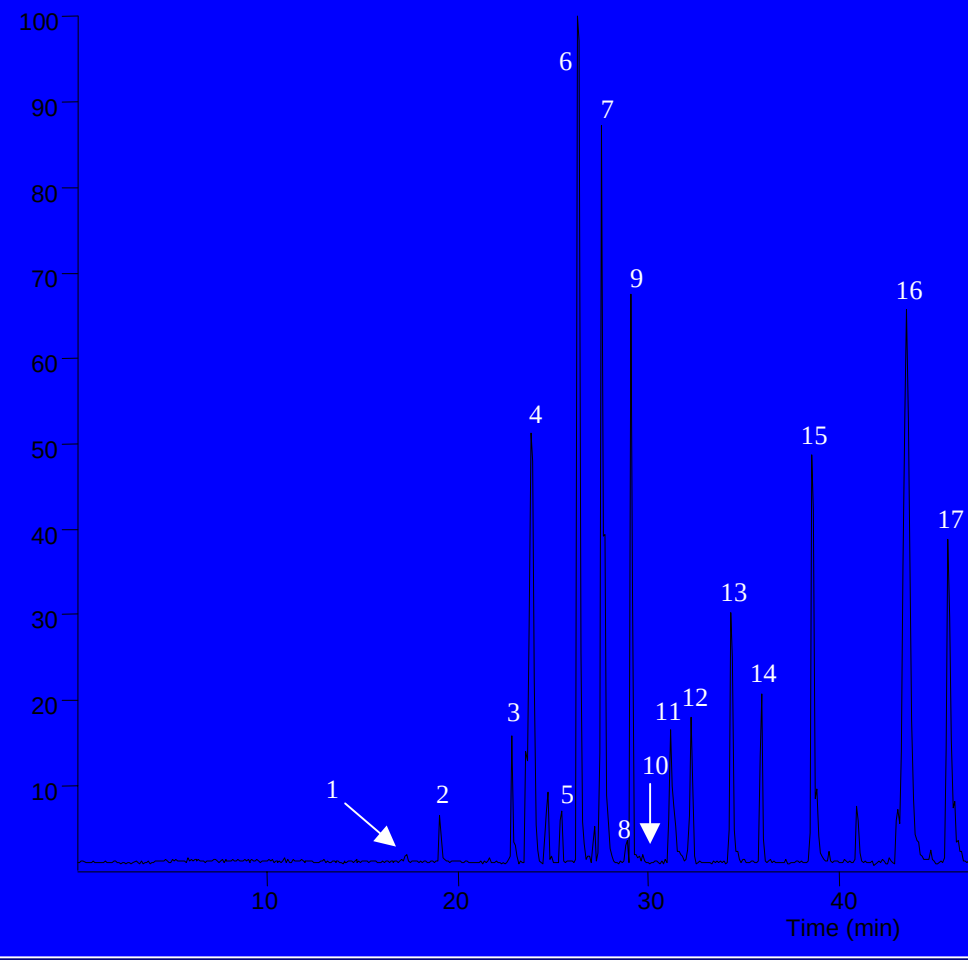
Tryptic Digest of Ovalbumin

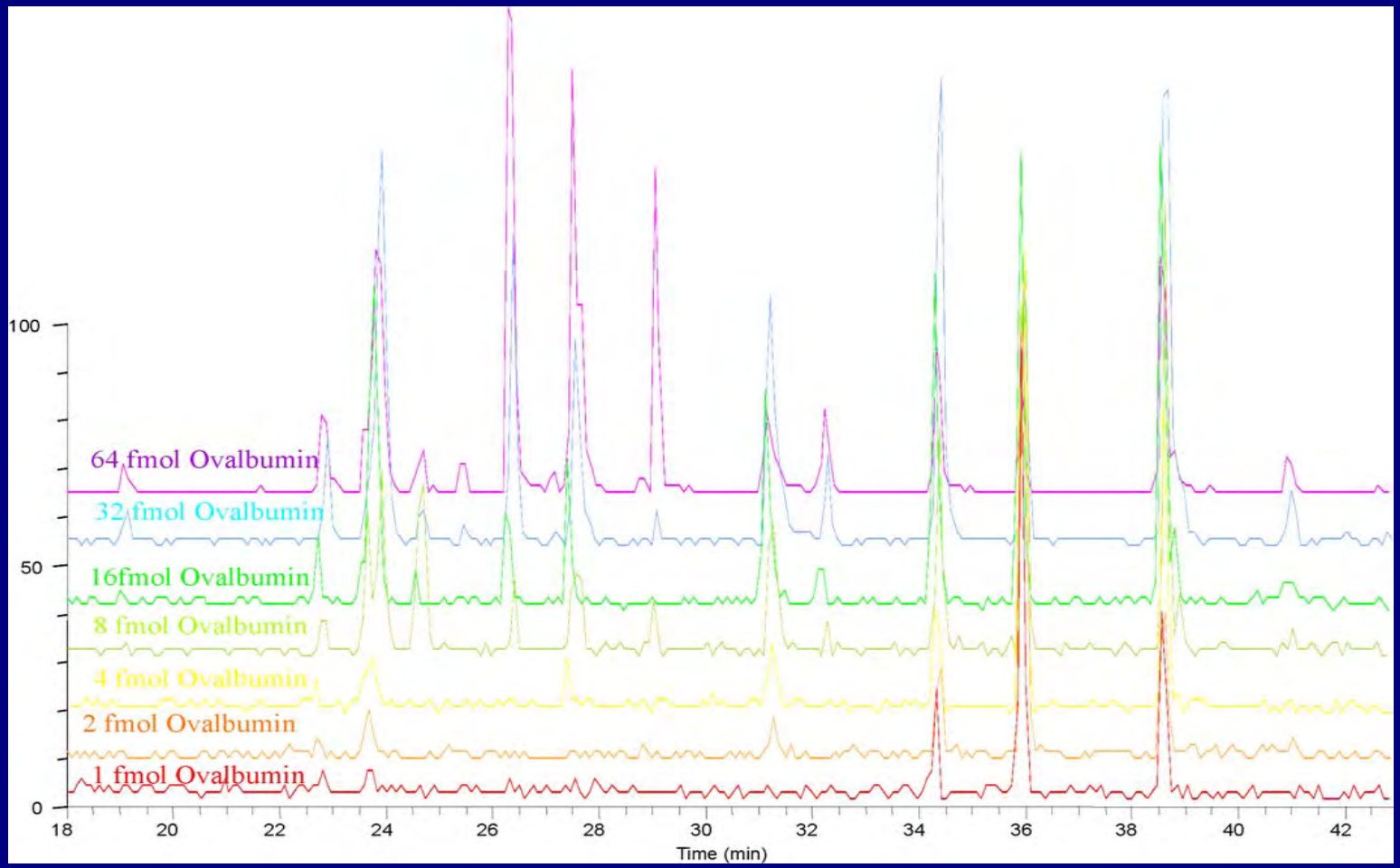


Ovalbumin Tryptic Digest

Sample: 64 fmol Ovalbumin Tryptic Digest
Column: SGE ProteCol™ (150µm x 100mm, C18, 3µm, 300Å)
Mobile Phase A: 0.1% Formic Acid
Mobile Phase B: 90% Acetonitrile (0.1% FA)
Gradient: 0% to 60%B in 40 min then 60% to 100%B in 5 min
Flow Rate: 550nL/min

1. VASMASEK
2. LYAEER
3. VYLPR
4. GLWEK
5. DEDTQAMPFR
6. AFKDEDTQAMPFR
7. LTEWTSSNVMEER
8. EVVGpSAEAGVDAASVSEEFR
9. GGLEPINFQTAADQAR
10. ELINSWVESQTNGIIR
11. HIATNAVLFFGR
12. DILNQITKPNDVYSFSLASR
13. VTEQESKPVQMMYQIG
14. PVQMMYQIGLFR
15. NVLQPSSVDSQTAMV
16. VHHANENIFYC*PIAIMSALAMVYLGAKDSTR
17. ILELPFASGTMSMLVLLPDEVSGLEQLESIINFEK

Sensitivity for Ovalbumin Digest



proteome systems
integrated proteome technology

SGE

Comparison of Sensitivities

Ovalbumin Load	"Market Leader" Column		SGE ProteCol	
	No of Peptides	% Coverage	No of Peptides	% Coverage
1 fmol	x	x	6	26.0
8 fmol	5	20.8	8	30.1
32 fmol	6	30.9	10	41.3
125 fmol	8	35.1	13	48.6
500 fmol	9	35.8	15	51.2
1 pmol	12	42.9	17	51.2

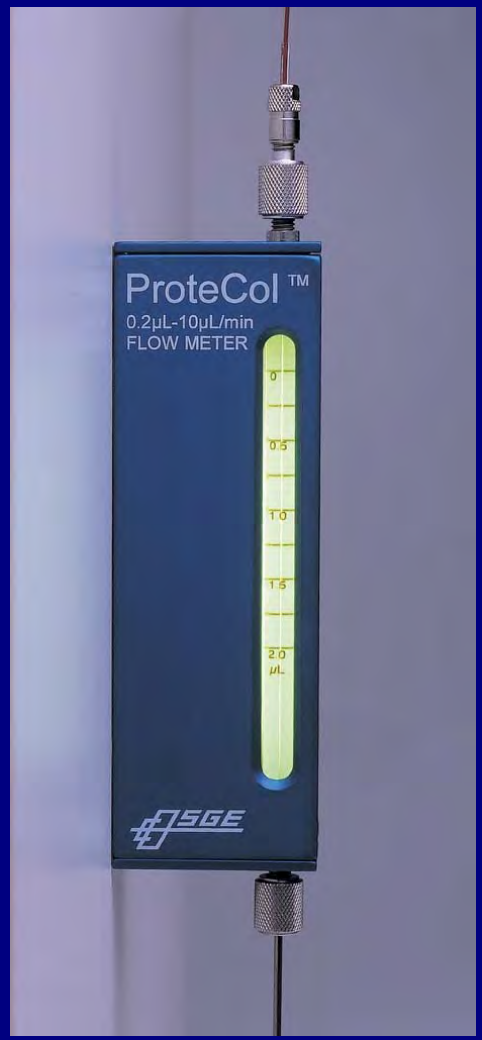
Column: 75µm x 100mm, C18, 3µm, 300Å
Flow Rate: 150nL/min

Column: 150µm x 100mm, C18, 3µm, 300Å
Flow Rate: 550nL/min

proteome systems
integrated proteome technology

SGE

ProteCol™ MicroFlow Meter



Specifications and features:

- Measures flow directly in a precisely calibrated syringe barrel
- Perfect companion to the flow splitter
- Two flow ranges available : 0.2 – 6 µl/min and 5 - 50 µl/min

Conclusions

- Peak dispersion or bandspreading lowers HPLC efficiency, resolution and mass sensitivity.
- Extremely small peak volumes in capillary LC creates a special challenge for making column connections.
- Dispersion outside of the column bed must be strictly minimized in order to separate complex mixtures with high resolution.
- Wide acceptance of capillary LC and LC/MS techniques will require continuing improvement in system, column and accessory designs.

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