Don’t Lose It: Troubleshooting Separation Changes

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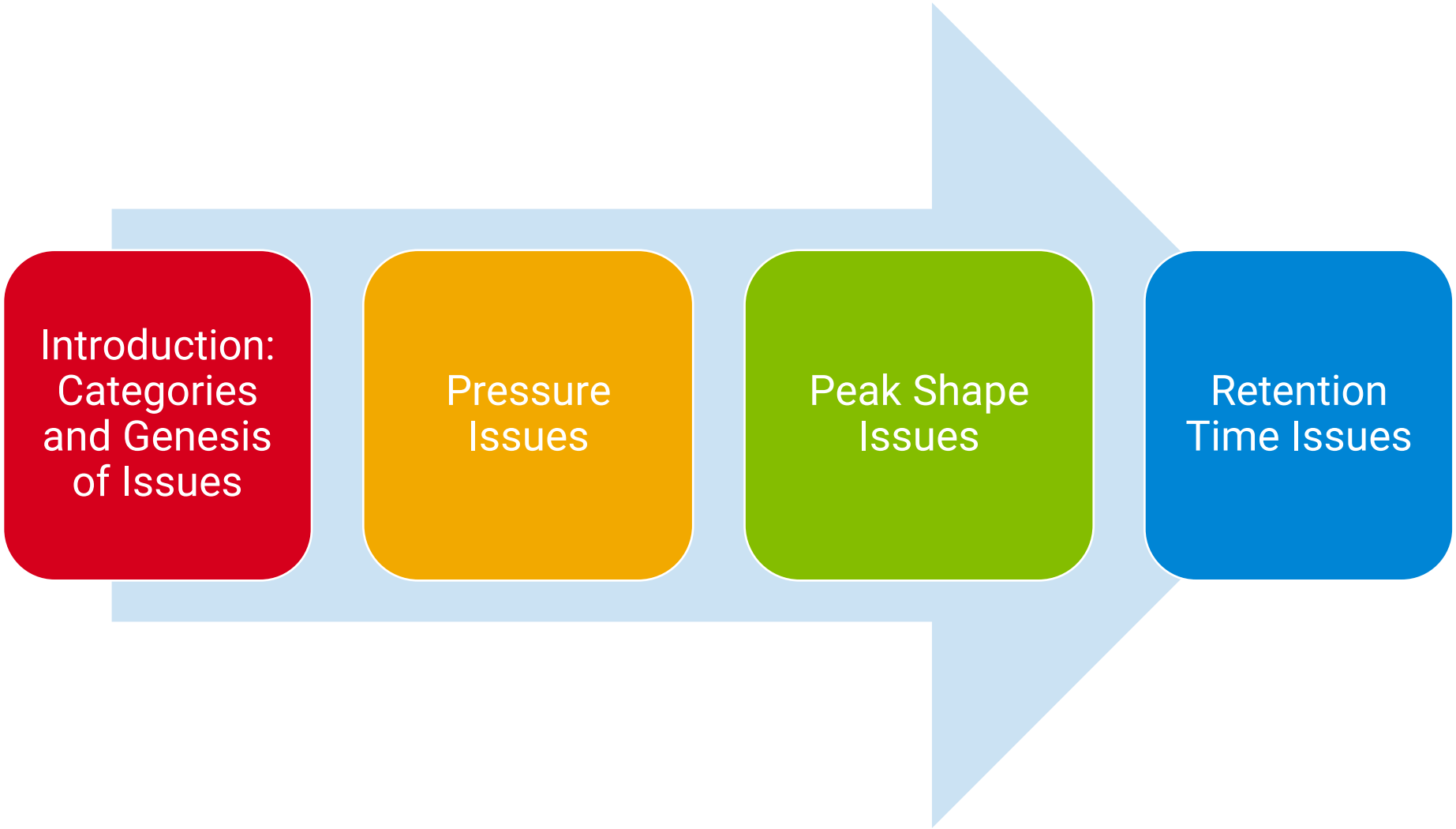
Troubleshooting Separation Changes



First Reaction When an HPLC Problem Arises

...the column has a problem

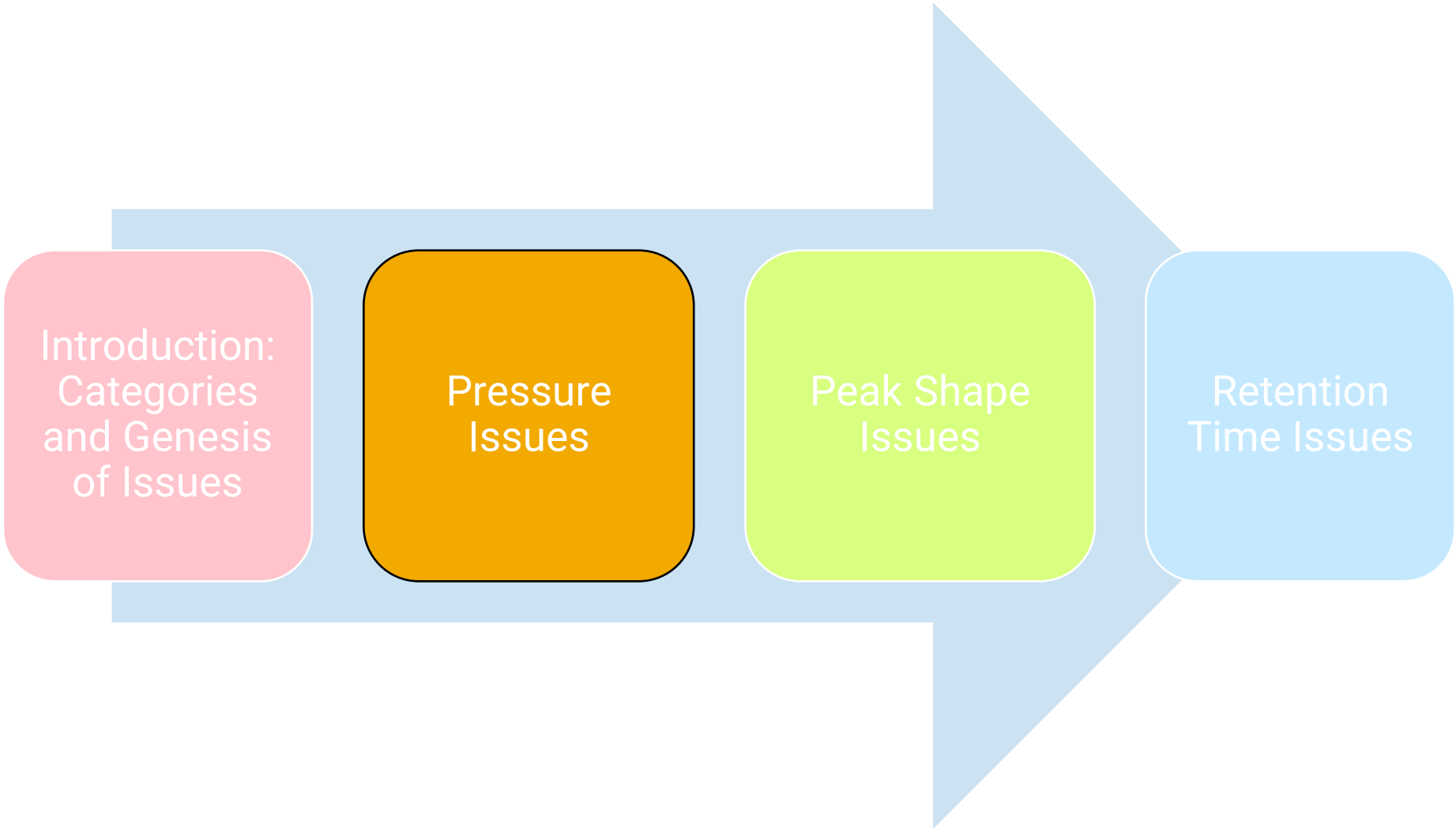
Experience of Typical HPLC Problems

- A third of problems are due to instrumental issues
  - External leaks
  - Internal leaks: Pump seals, inlet, and outlet valves
  - Injector maintenance: Rotor seal, needle seat
  - Poor connections
  - Data system not optimized

- A third of problems come from column issues
  - Plugging, increasing pressure
  - Loss of bonded phase
  - Voids, settling

- A third of issues come from method problems
  - Mobile phase incorrect (for example, wrong pH, buffer concentration, solvent)
  - Inadequate sample preparation
  - Borderline ruggedness

Troubleshooting Separation Changes



Pressure Issues

Column Observations	Potential Problems
High pressure	- Clogged frit
	- Clogged packing
	- Blockage in HPLC flow path
Low pressure	- Leak
	- Flow incorrect
Fluctuating pressure	- Pump not operating correctly
	- Air in system

Determining the Cause and Correcting High Back Pressure

Check pressure with/without column

- Replace column with ZDV union and recheck pressure. If it is still high, then the clog is in the flow path.

If pressure is only high with column in place:

Rinse or backflush column (remove detector from flow path)

- Eliminate column contamination and plugged packing
- Remove precipitate from sample or buffer

Install new column

Eliminate pressure issues

- Check buffer and sample solubility
- Add a disposable 0.2, 0.5, or 2 µm inline filter to system.

Column Cleaning

Flush with stronger solvents than your mobile phase.

Reversed-phase solvent choices in order of increasing strength

Use at least 25 mL of each solvent for analytical columns

This is time consuming and often performed offline

- Mobile phase without buffer salts
- 100% Methanol
- 100% Acetonitrile
- 75% Acetonitrile:25% isopropanol
- 100% Isopropanol
- 100% Methylene chloride*
- 100% Hexane*

Must reverse to re-equilibrate

*Tip: When using either hexane or methylene chloride, the column must be flushed with isopropanol before returning to your reversed-phase mobile phase.

The Trick: Prevention Techniques - A Better Choice

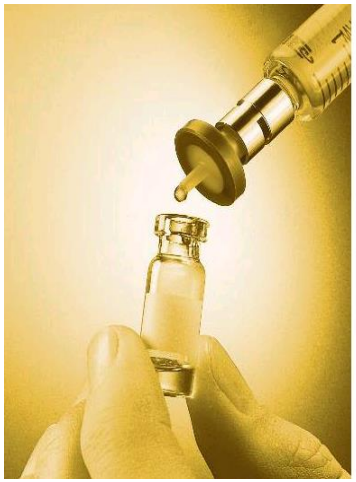
- Use column protection
  - Inline filters
  - Guard columns
- Filter samples
- Filter buffered mobile phases

Easy

- Sample cleanup (SPE)
- Appropriate column flushing

Not as easy

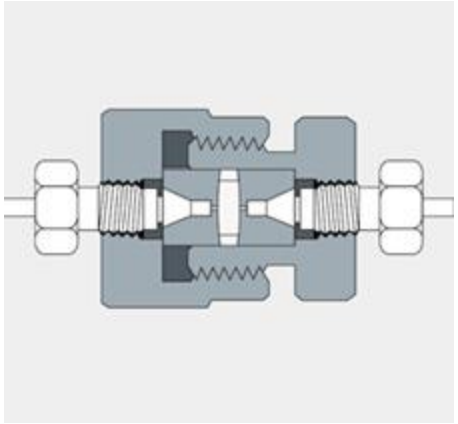
Inexpensive Filters Prevent Column Frit Plugging



Regenerated cellulose (RC) Recommended

- Universal hydrophilic membrane, compatible with most solvents – aqueous and organic
- High purity, extremely low extractables and binding
- More uniform surface
- *Different to other cellulose filters*

- Inline filters easy to use and replace
- Frits available in 0.2, 0.5 and 2.0 µm porosity
- Much less expensive than a guard or column



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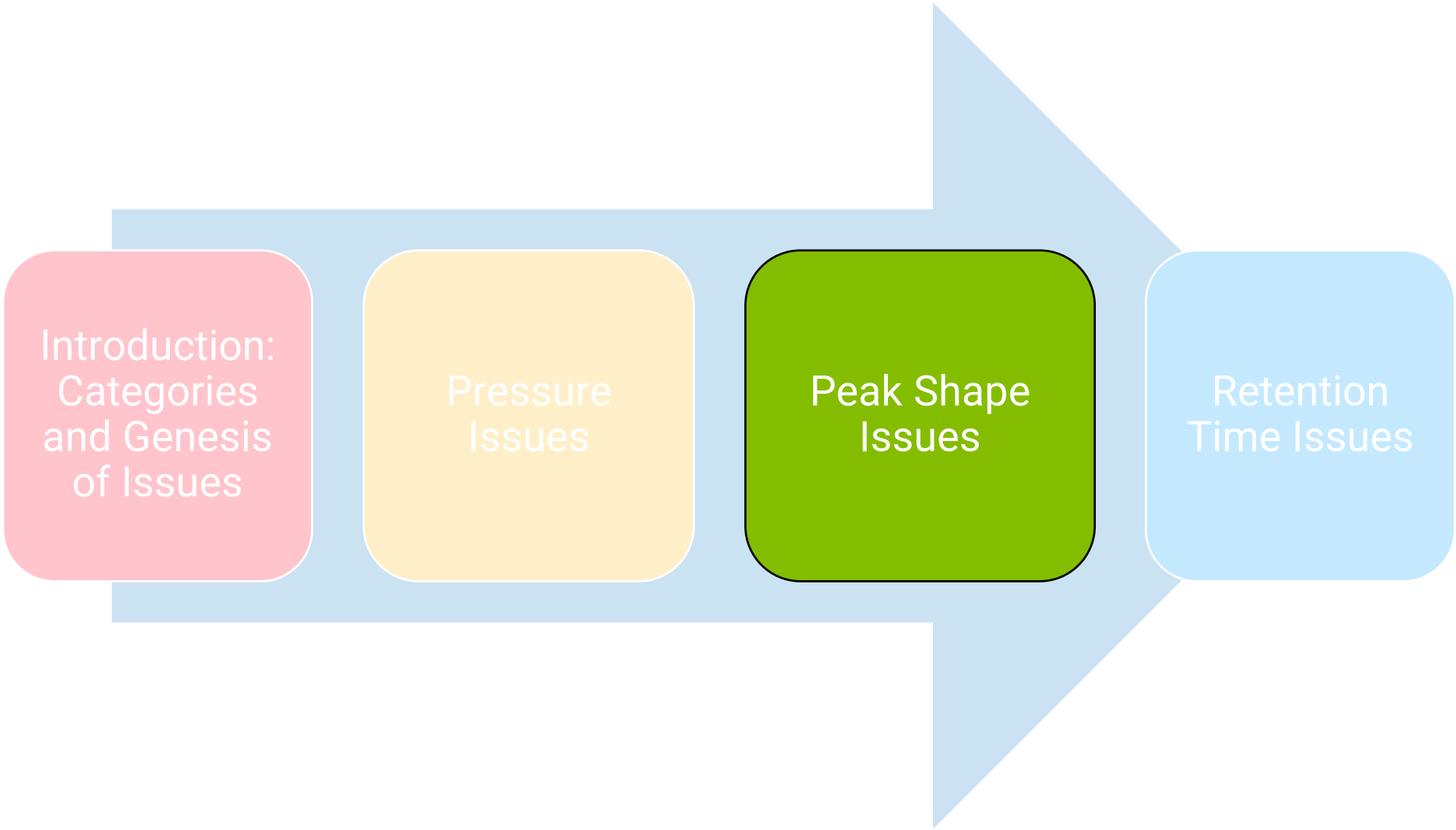


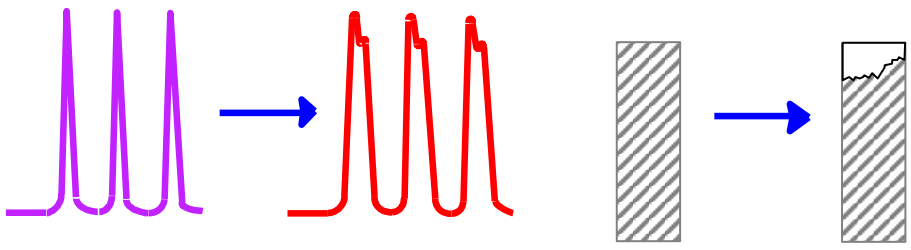
What are Common Peak Shape Issues?

1. Peak tailing/fronting
2. Broad peak
3. Split peaks

- Many peak shape issues are also combinations, for example, broad and tailing or tailing with increased retention
- Symptoms do not necessarily affect all peaks in the chromatogram
- Each of these problems can have multiple causes

Peak Splitting Caused By Disrupted Sample Path

- Flow path disrupted by void
- Sample allowed to follow different paths through column
- Poorly packed bed settles in use
- High pH dissolves silica

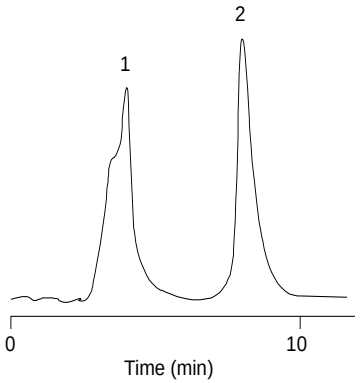


Tip: A similar effect can be caused by partially plugged frit.

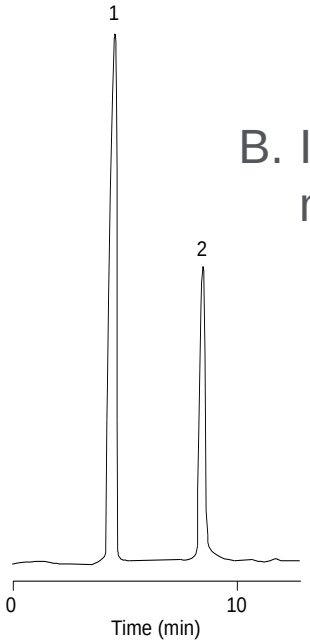
Split Peaks from Injection Solvent Effects

Column: StableBond SB-C8, 4.6 x 150 mm, 5 µm Mobile phase: 82% H_2O : 18% ACN
Injection volume: 30 mL Sample: 1. Caffeine 2. Salicylamide

A. Injection solvent 100% acetonitrile



B. Injection solvent mobile phase

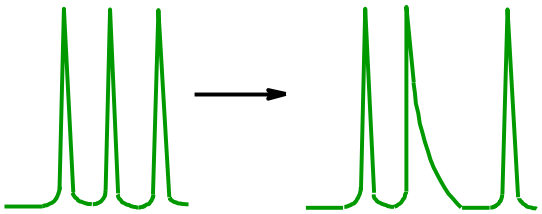


Tip: Injecting in a solvent stronger than the mobile phase can cause peak shape problems, such as peak splitting or broadening.

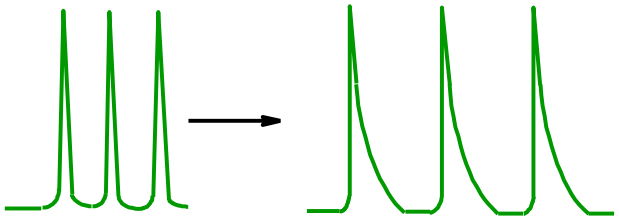
Trick: Keep organic concentration in sample solvent ≤ mobile phase

Peak Shape: Tailing Peaks

Symmetry > 1.2



Normal Tailing



Normal Tailing

Common causes

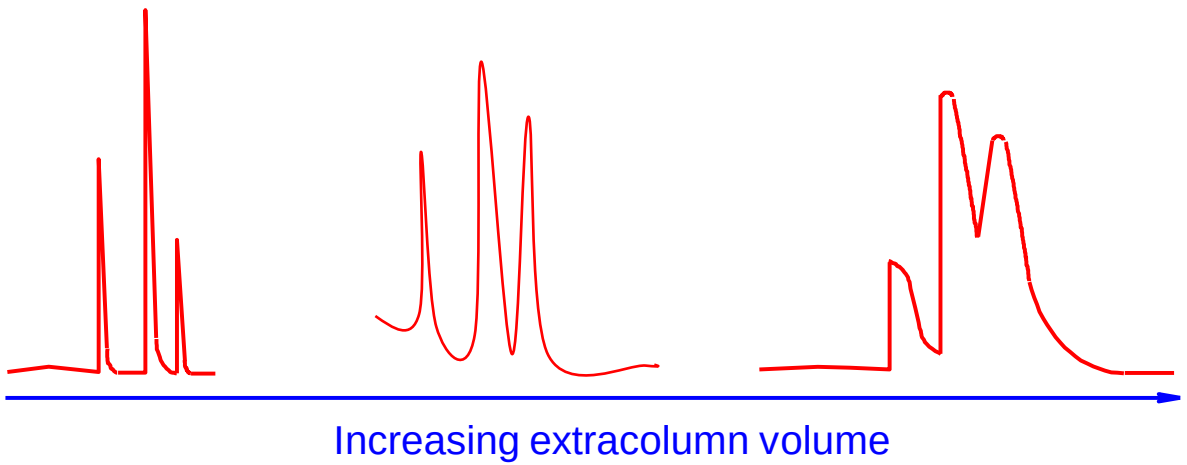
- Some peaks tail
  - Secondary - retention effects
  - Residual silanol interactions
  - Small peak eluting on tail of larger peak
- All peaks tail
  - Extracolumn effects
  - Build up of contamination on column inlet
  - Heavy metals
  - Column has aged and gone “bad”

Peak Tailing, Broadening and Loss of Efficiency

May be caused by:

- Column “secondary interactions”
- Column contamination
- Column aging
- Column loading
- Extracolumn effects

Extracolumn Dispersion (Volume)



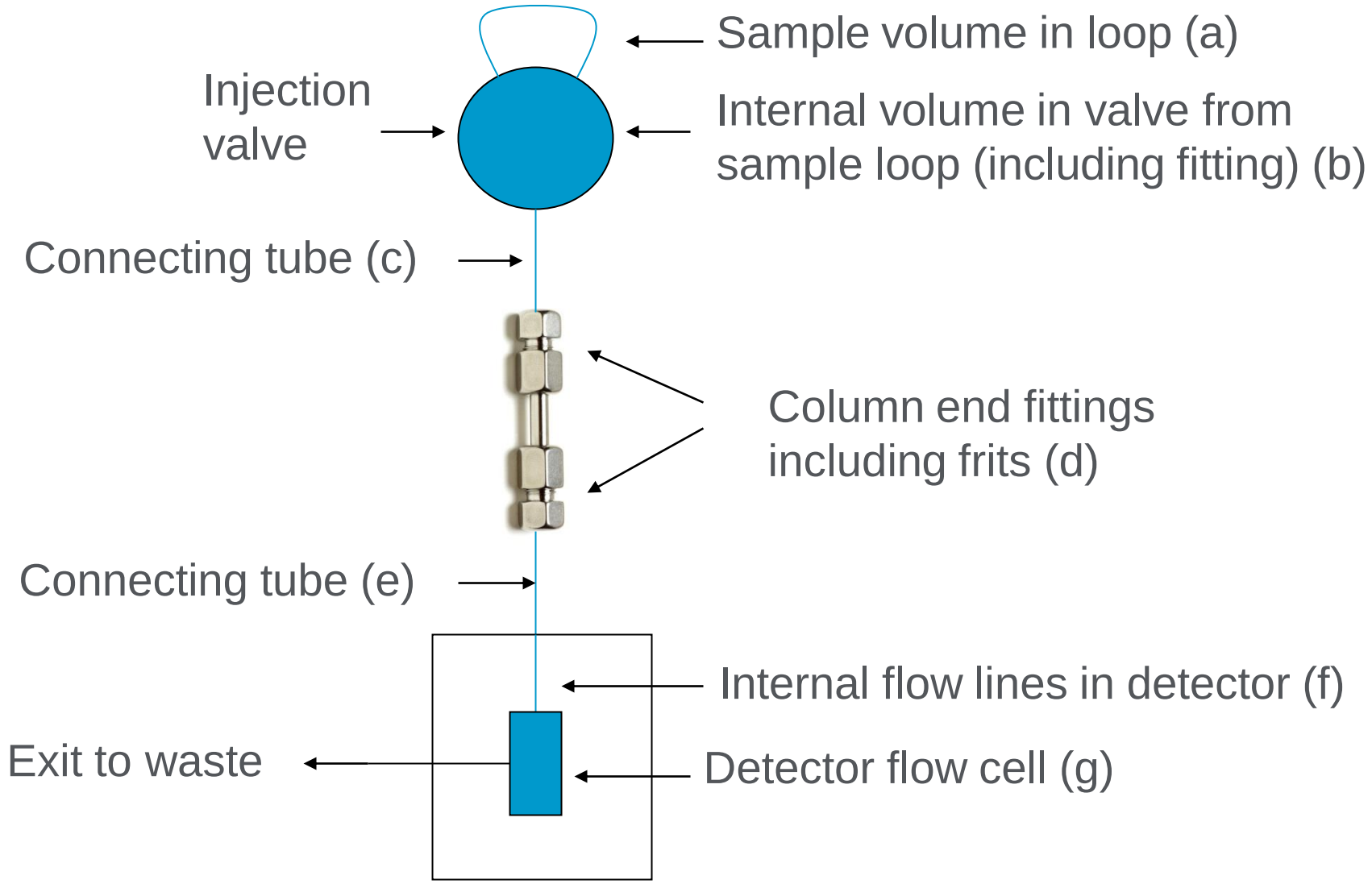
Use short, small internal diameter tubing between the injector and the column, and between the column and the detector.

Make certain all tubing connections are made with matched fittings.

Use a low-volume detector cell

Inject small sample volumes

Extracolumn Volumes in HPLC Sample Flow System



Tip: Poorly Made HPLC System Connections Can Cause Peak Broadening

The system has been optimized and:

- All tubing lengths are minimum
- Smallest diameter tubing used
- Proper flow cell volume

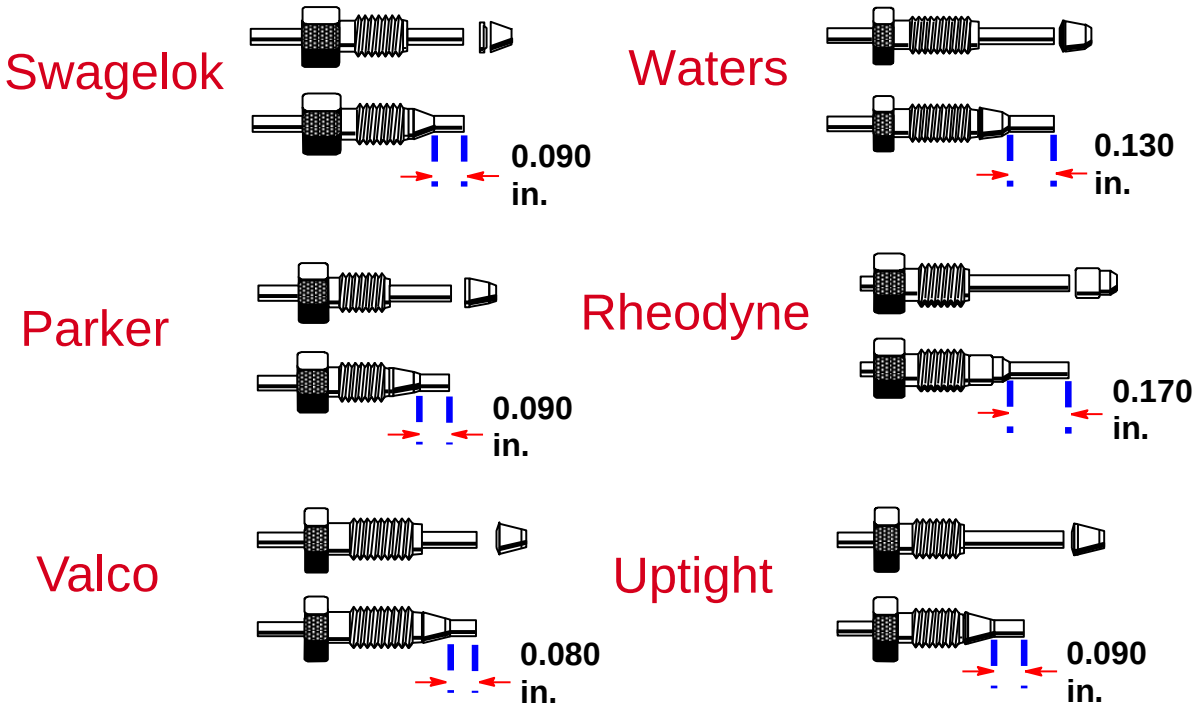
Symptom still seems to have too much extracolumn volume

What is wrong?

Have you made the connections properly?

Column Connectors Used in HPLC

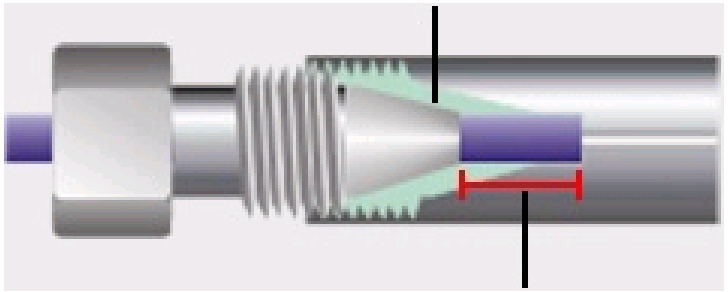
Troubleshooting LC Fittings, Part II. J. W. Dolan and P. Upchurch. LC/GC Magazine 6:788 (1988)



What Happens if Connections are Poorly Made?

Wrong, too long

Ferrule cannot seat properly



X

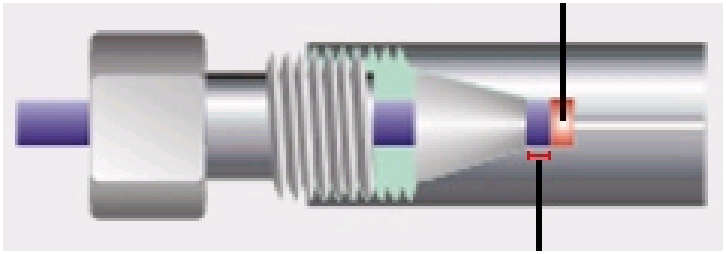
If dimension X is too long, leaks will occur

These poor connections cause:

- Poor efficiency
- Peak tailing
- Leaking

Wrong, too short

Mixing chamber



X

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

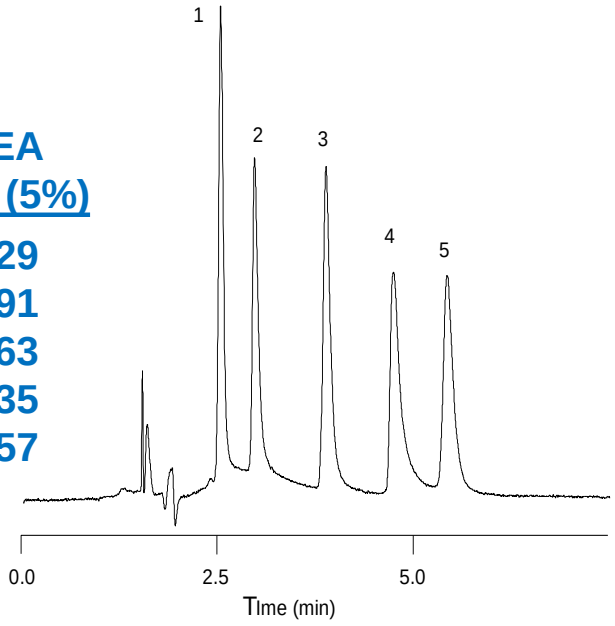
Peak Tailing

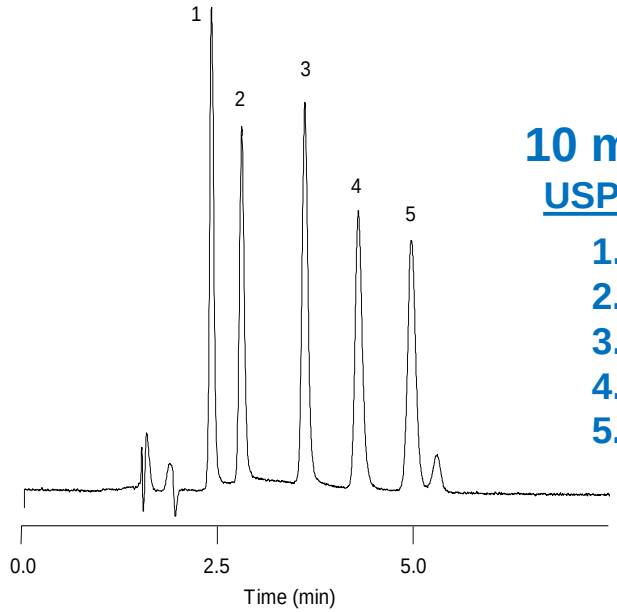
Identifying column “secondary interactions”

Column: Alkyl-C8, 4.6 x 150 mm, 5 µm Mobile phase: 85% 25 mM Na_2HPO_4 pH 7.0 : 15% ACN Flow rate: 1.0 mL/min
Temperature: 35 °C Sample: 1. Phenylpropanolamine 2. Ephedrine 3. Amphetamine 4. Methamphetamine 5. Phenteramine

No TEA
USP TF (5%)

1. **1.29**
2. **1.91**
3. **1.63**
4. **2.35**
5. **1.57**





10 mM TEA
USP TF (5%)

1. **1.19**
2. **1.18**
3. **1.20**
4. **1.26**
5. **1.14**

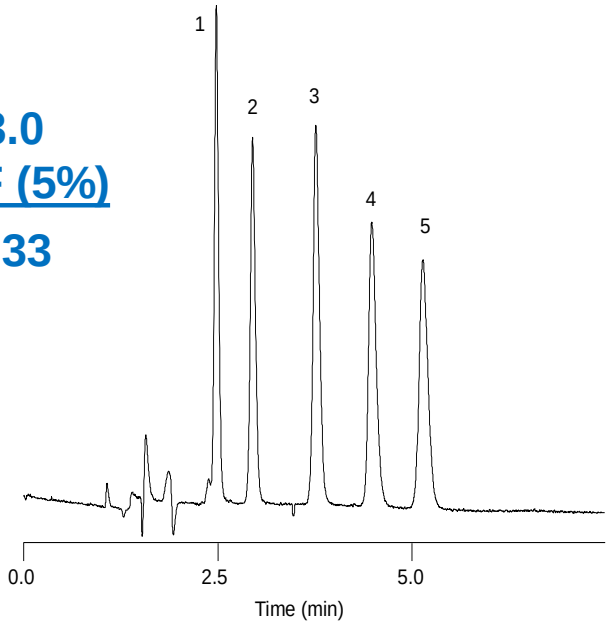
Tip: Mobile phase modifier (TEA = triethylamine) competes with sample molecule for surface ion exchange sites at mid-range pH values.

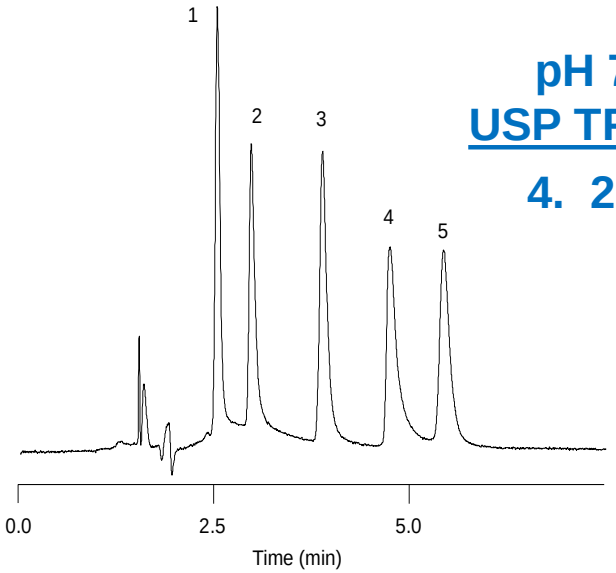
Peak Tailing

Low pH minimizes secondary interactions for amines

Column: Alkyl-C8, 4.6 x 150 mm, 5 µm Mobile phase: 85% 25 mM Na_2HPO_4 : 15% ACN Flow rate: 1.0 mL/min
Temperature: 35 °C Sample: 1. Phenylpropanolamine 2. Ephedrine 3. Amphetamine 4. Methamphetamine 5. Phenteramine

pH 3.0
USP TF (5%)
4. 1.33





pH 7.0
USP TF (5%)
4. 2.35

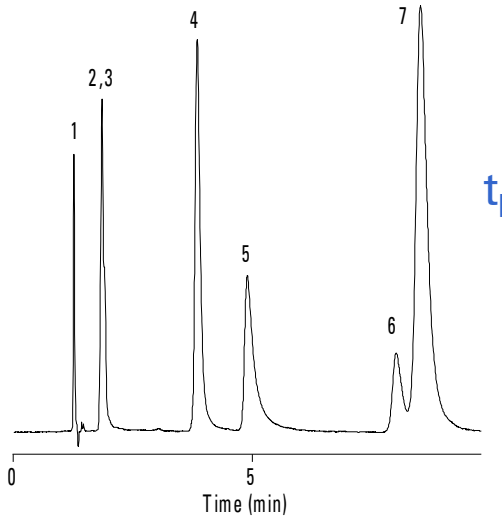
Tip: Reducing mobile phase pH reduces interactions with silanols and peak tailing.

Peak Tailing

High pH minimizes secondary interactions for amines

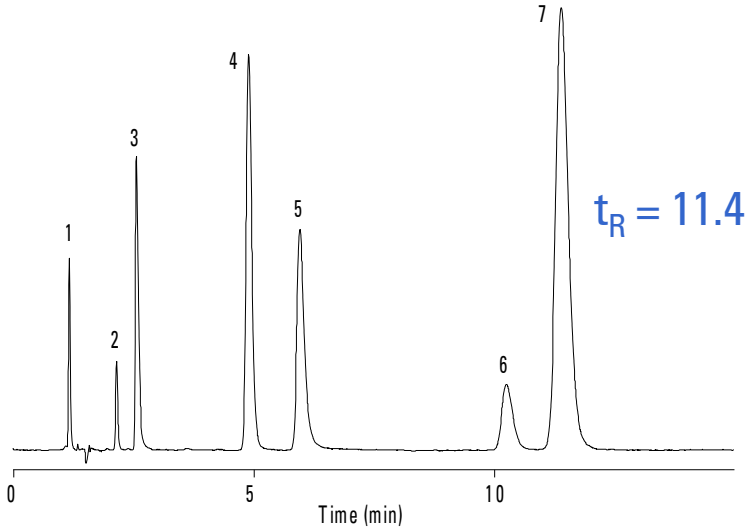
Column: ZORBAX Extend-C18, 4.6 x 150 mm, 5 µm Mobile phase: See Below Flow rate: 1.0 mL/min Temperature: RT
Detection: UV 254 nm
Sample: 1. Maleate 2. Scopolamine 3. Pseudoephedrine 4. Doxylamine 5. Chlorpheniramine 6. Triprolidine 7. Diphenhydramine

pH 7
30% 20 mM Na_2HPO_4
70% MeOH



$t_R = 8.5$

pH 11
30% 20 mM TEA
70% MeOH



$t_R = 11.4$

Peak shape and retention of this sample of basic compounds improves at high pH where column has high IEX activity. Why?

Peak Tailing

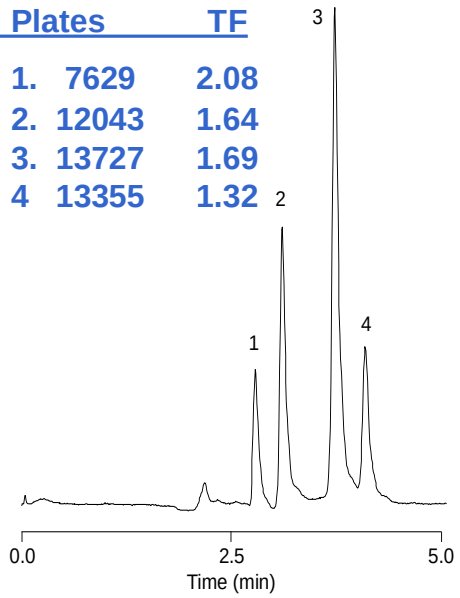
Column contamination

Tip: Quick test to determine if column is dirty or damaged

Trick: Reverse column and run sample –If Improved, possible cleaning will help. If there's so improvement the column damaged and needs to be replaced

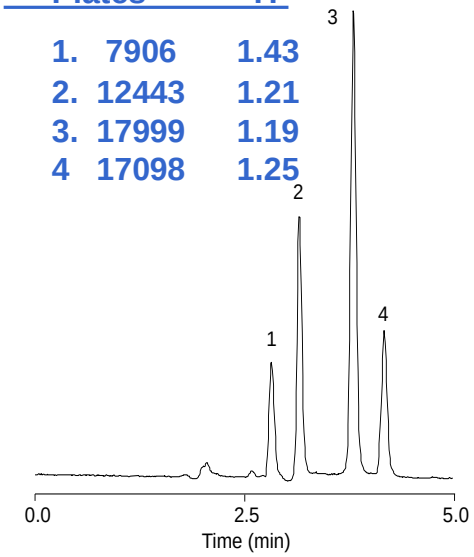
QC test forward direction

	Plates	TF
1.	7629	2.08
2.	12043	1.64
3.	13727	1.69
4	13355	1.32



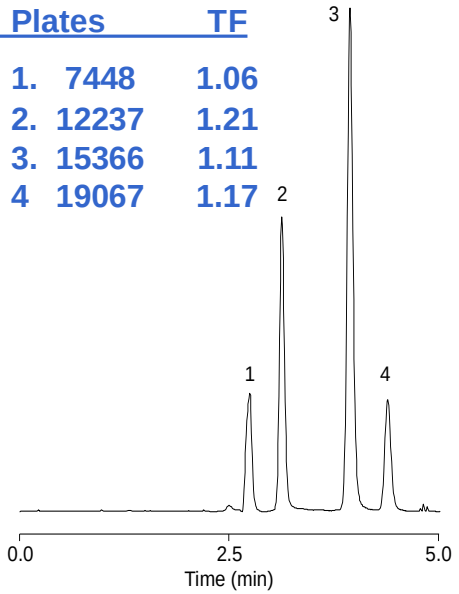
QC test reverse direction

	Plates	TF
1.	7906	1.43
2.	12443	1.21
3.	17999	1.19
4	17098	1.25



QC test after cleaning 100% IPA, 35 °C

	Plates	TF
1.	7448	1.06
2.	12237	1.21
3.	15366	1.11
4	19067	1.17



Column: StableBond SB-C8, 4.6 x 250 mm, 5 µm Mobile phase: 20% H_2O : 80% MeOH Flow rate: 1.0 mL/min
Temperature: R.T. Detection: UV 254 nm Sample: 1. Uracil 2. Phenol 3. 4-Chloronitrobenzene 4. Toluene

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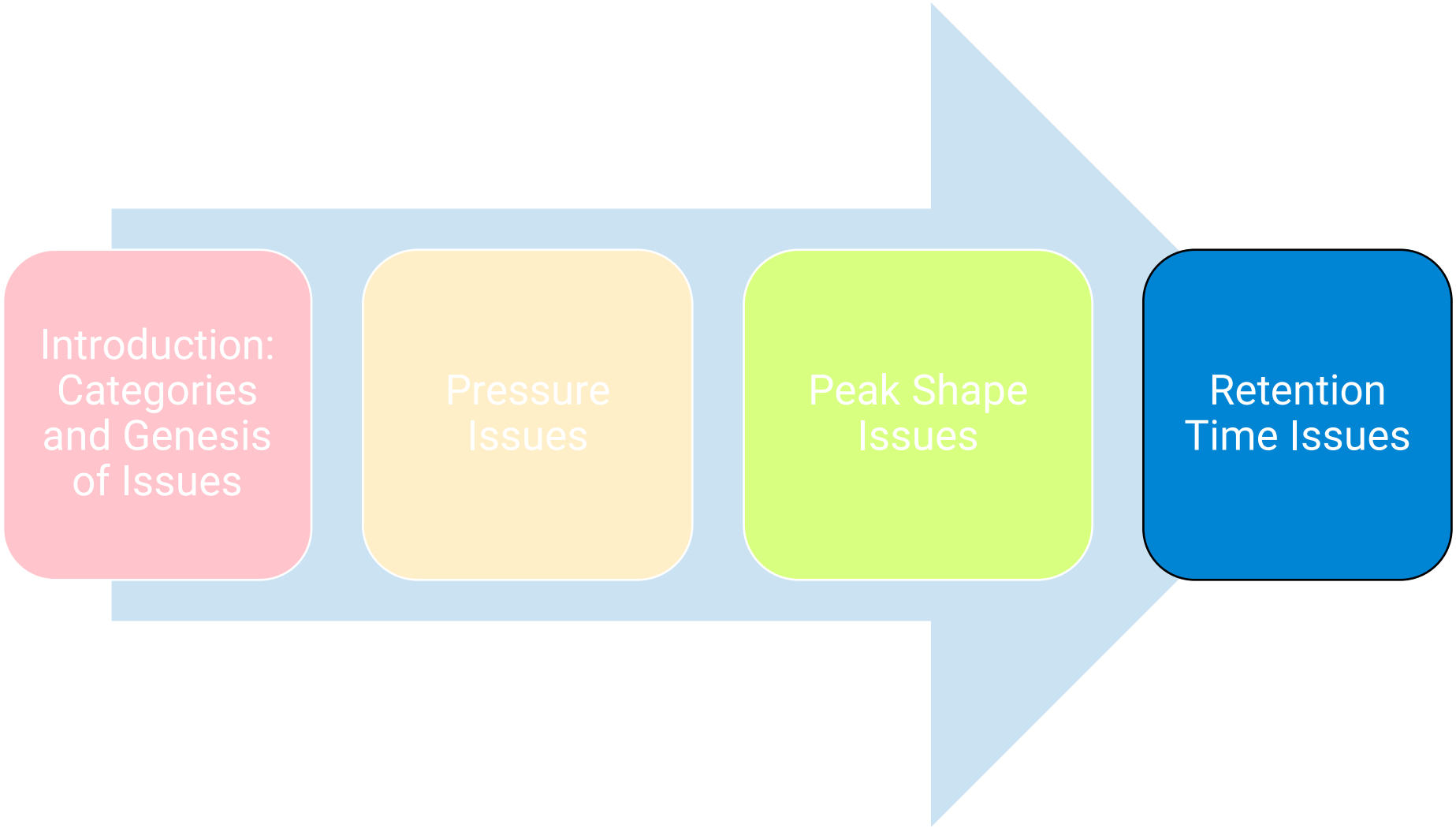

Separation Conditions that Cause Changes in Retention*

Flow rate	**+/- 1%**	**+/- 1% Tr**
Temp	**+/- 1 °C**	**+/- 1 to 2% Tr**
%Organic	**+/- 1%**	**+/- 5 to 10% Tr**
pH	**+/- 0.01%**	**+/- 0 to 1% Tr**

*excerpt from “Troubleshooting HPLC Systems”, J. W. Dolan and L. R. Snyder, p 442.

Changes in Retention (k) – Same Column, Over Time

May be caused by:

1. Column aging
2. Column contamination
3. Insufficient column equilibration
4. Poor column/mobile phase combination
5. Change in mobile phase
6. Change in flow rate
7. Change in column temperature
8. Other instrument issues (for example, different gradient delay volumes)

Delay Aging and Contamination Effects with Good Column Practices

- Filter buffers
- Investigate the effects of sample solvent on solubility and separation.
- Pretreat samples that contain strongly retained components of no interest.
- Awareness of column packing limits
  - pH
  - Temperature
  - Chemical compatibility
- Use fresh aqueous solutions and consider the use of a bio-stat (sodium azide).
- Flush column periodically with strong solvent
- To store the column, purge buffers and leave it in appropriate solvent (ACN).
- Avoid physically mishandling columns by banging, dropping, or over tightening fittings.

Retention Time Changes Due to Instrument Issues

5. Change in mobile phase
6. Change in flow rate
7. Change in column temperature
8. Other instrument issues (for example, different gradient delay volumes)

Contact Agilent Chemistries and Supplies Technical Support

1-800-227-9770 option 3, option 3:

Option 1 for GC and GC/MS columns and supplies

Option 2 for LC and LC/MS columns and supplies

Option 3 for sample preparation, filtration and QuEChERS

Option 4 for spectroscopy supplies

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Questions?