Waters

Troubleshooting Common HPLC Problems

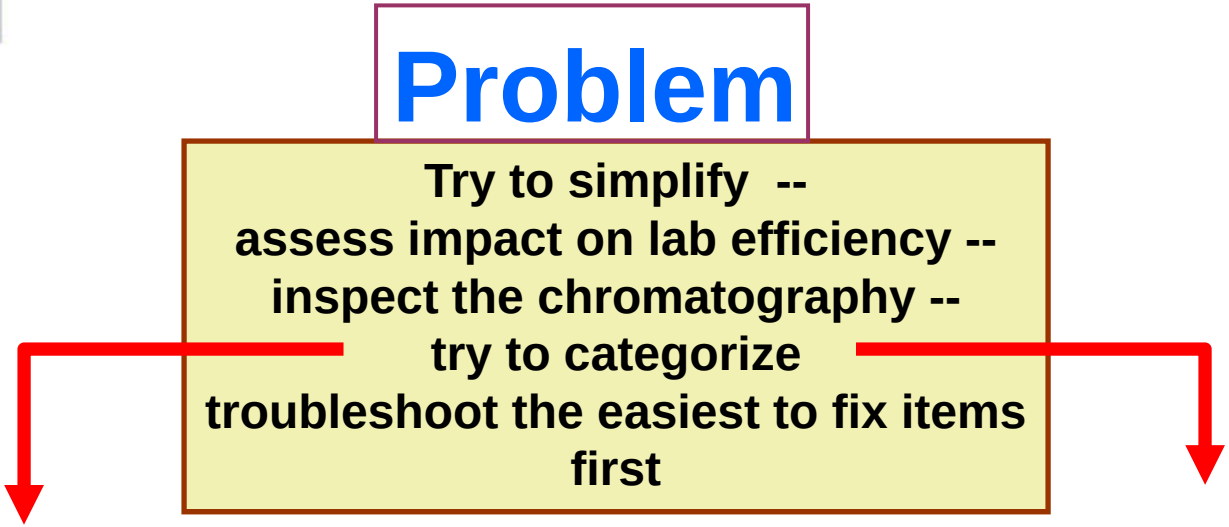
Waters

Potential Sources of Chromatographic Problems

- Mobile Phase
- Injector
- In-Line Filter
- Column
- Detector
- Sample
- Pump
- Guard Column
- Connecting Tubing and Fittings
- Integrator/Recorder Software

Scientist/Analyst -- need for logical approach to save time

Troubleshooting Strategy



CHEMISTRY

- COLUMN
  - GUARD COLUMN
- MOBILE PHASE
- SAMPLE/VIALS

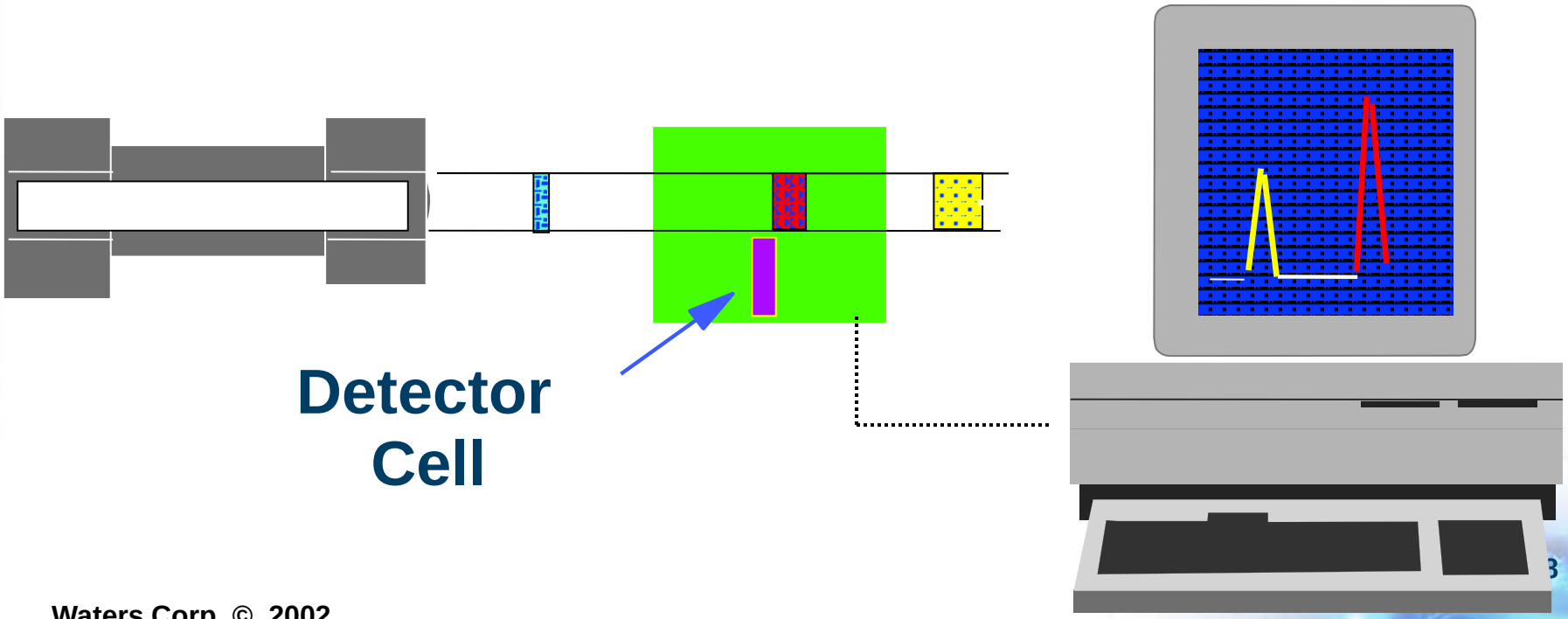
MECHANICAL

- PUMP
- INJECTOR
- DETECTOR
- DATA COLLECTION
- BAND SPREADING/ CONNECTIONS
- COLUMNS
- SAMPLE VIALS

Waters

How to Categorize -- Inspect Chromatogram

- How do you get sharp peaks with excellent resolution?
  - Well Shaped Bands -- Well Separated
  - (Good Mechanical And Chemical Performance)



Why Do You Get Distorted Peaks?

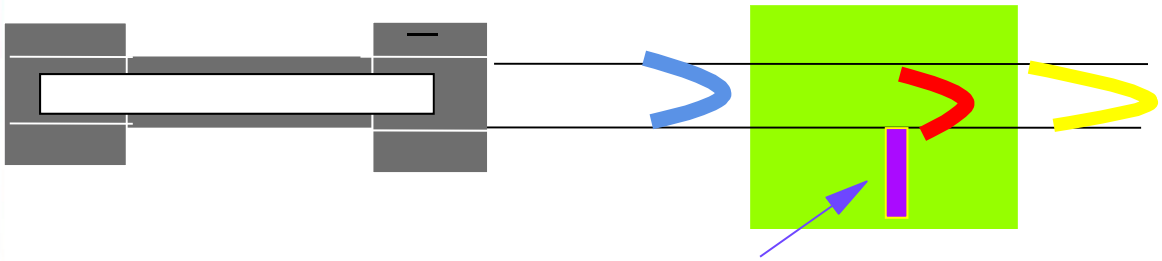
- **Why do you get all distorted peaks?**
  - **Distorted Bands –**

Mechanical Problem

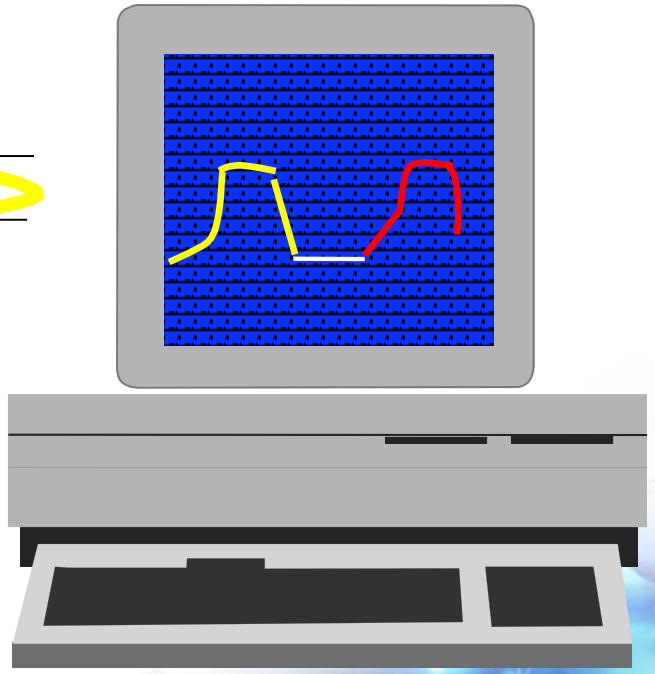
- **Injector**
- **Voided Column**
- **Poor Connections**

Chemical Problem

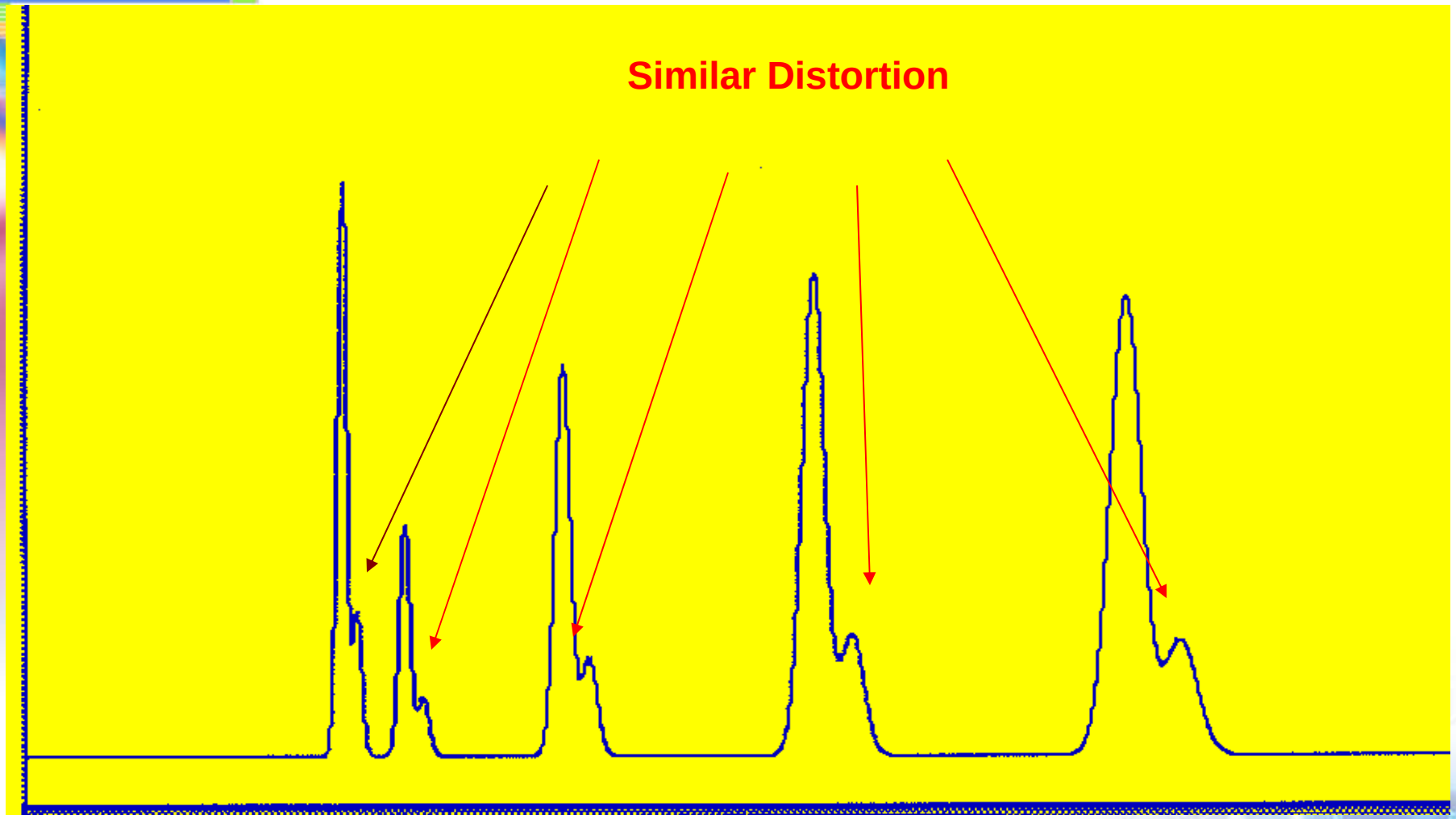
- Too Strong Sample Solvent



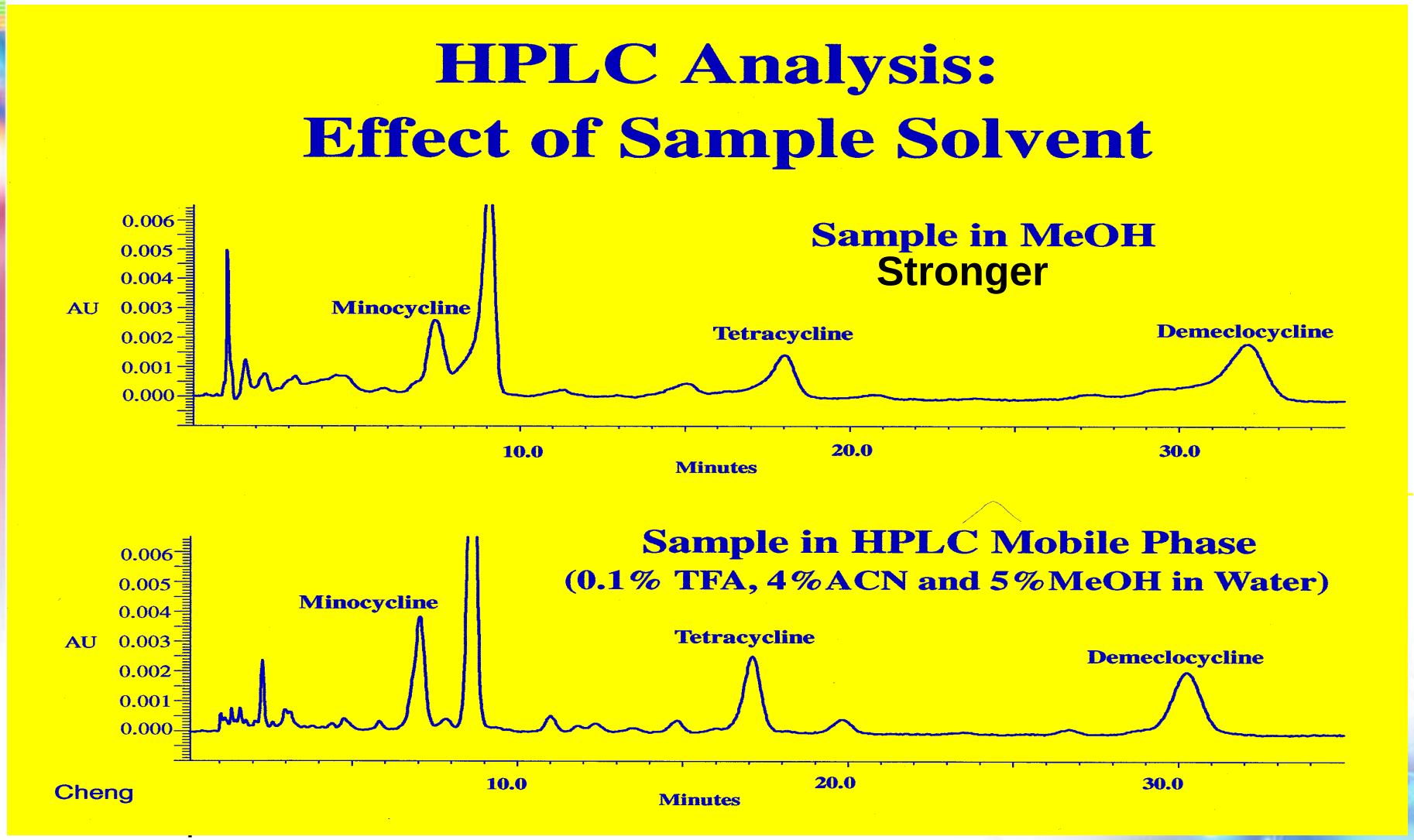
Detector Cell



All Peaks Distorted -- Mechanical Problem

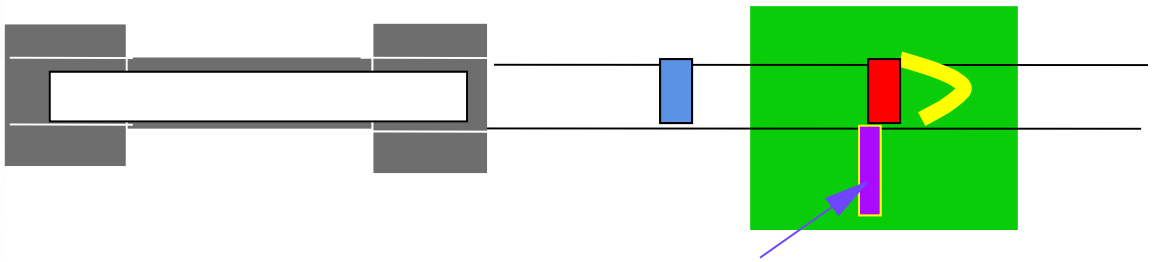


All Peaks Distorted – Chemical Problem
Incorrect Sample Solvent – STRONGER than mobile phase

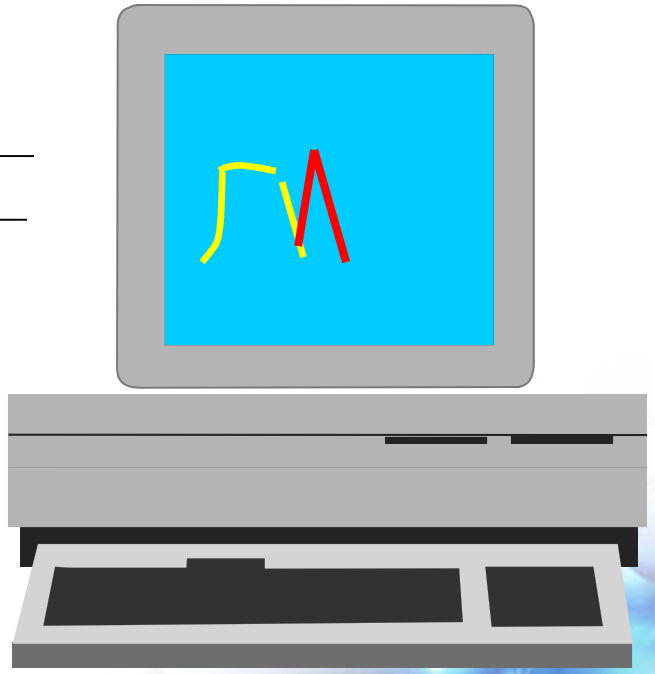


Why Do You Get One/Some Distorted Peaks?

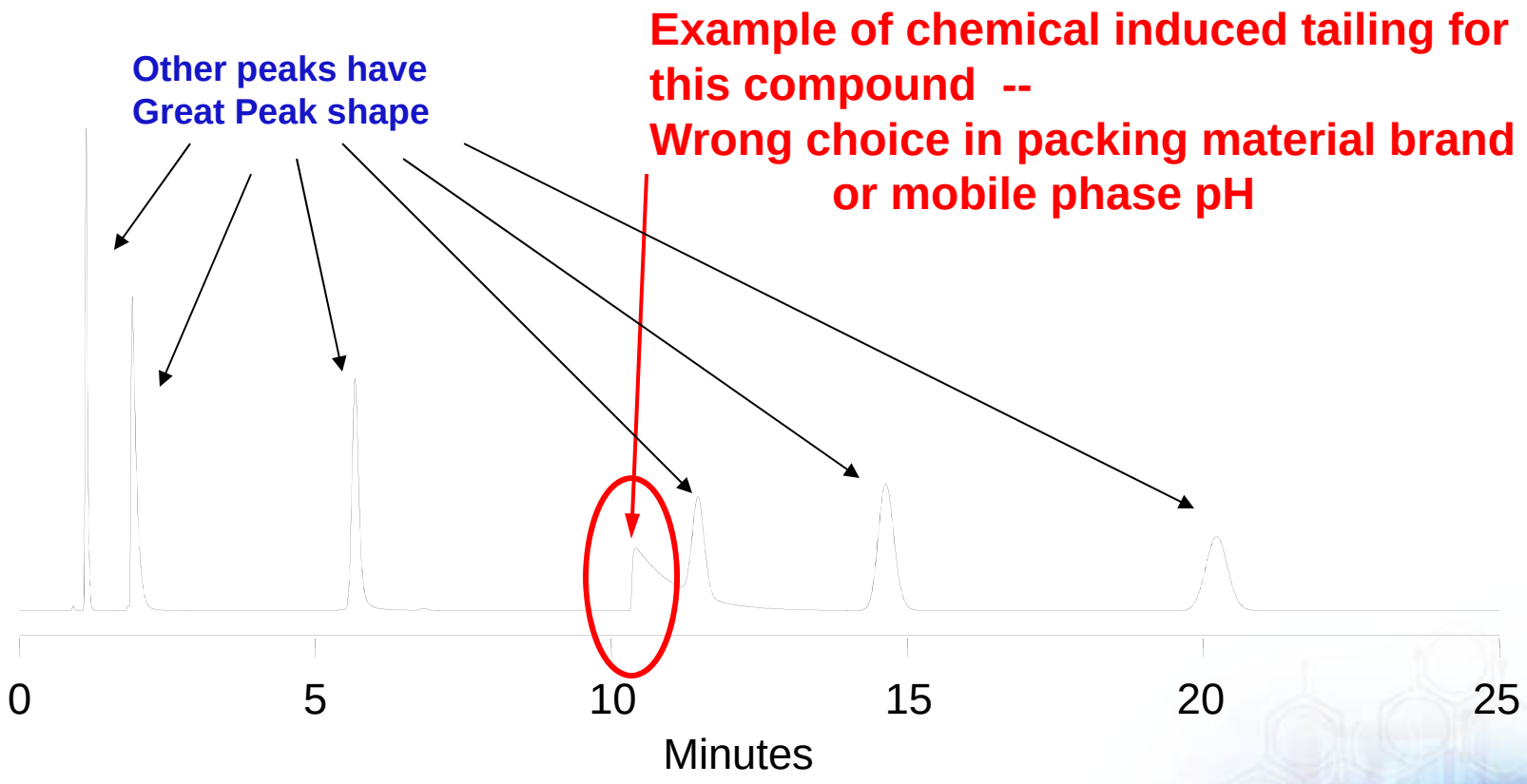
- Why do you get one or some distorted peaks?
  - Distorted Band – Chemical Problem
    - Cation exchange of one analyte to particle surface



Detector Cell



Great Peak Shape for Some Peaks, but Others Have Poor Peak Shape --- Chemical Problem (Not Mechanical)



Waters

Mechanical

* Extra-Column (Non – Column) Band Spreading

- Injection Volume
- Injector (seal problem)

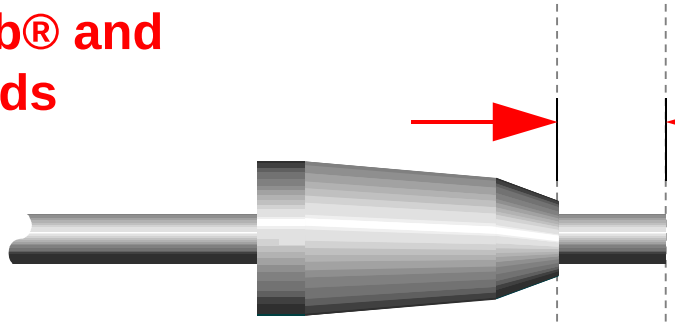
} •Remember: the band is initially created by the injector

- Connecting Tubing
  - Injector to Column
  - Column to Detector
- End-fittings and Frits
- Detector Volume

* Column Itself
  - •Voiding
  - •Plugged frits

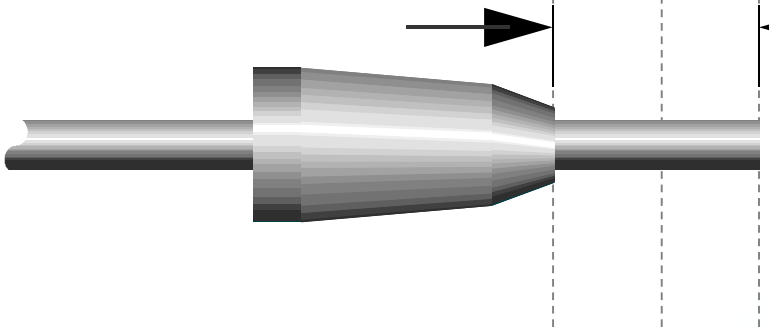
Column Installation and Connections - Mechanical

Waters Spherisorb® and many other brands



0.090" Parker Style

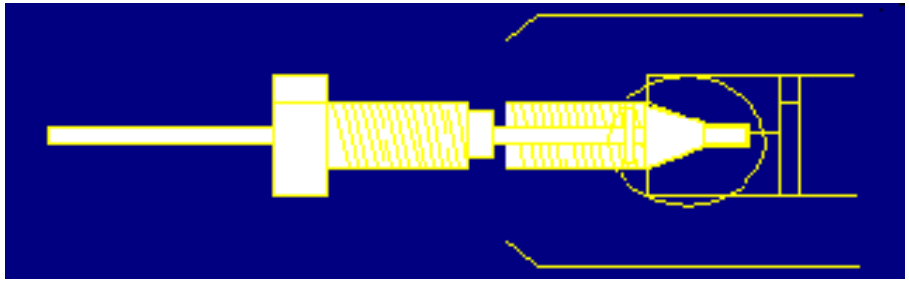
Other Waters® Columns



0.130" Waters Style

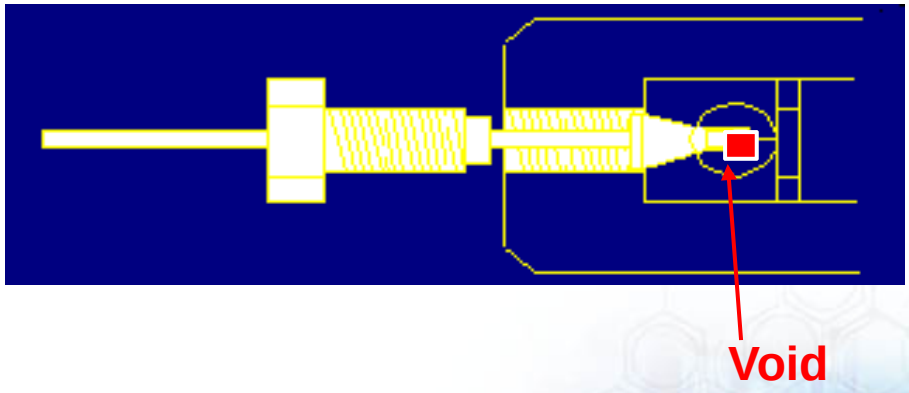
Installation -- Mechanical

- Make sure column connected correctly
- Make sure nut and ferrule are seated



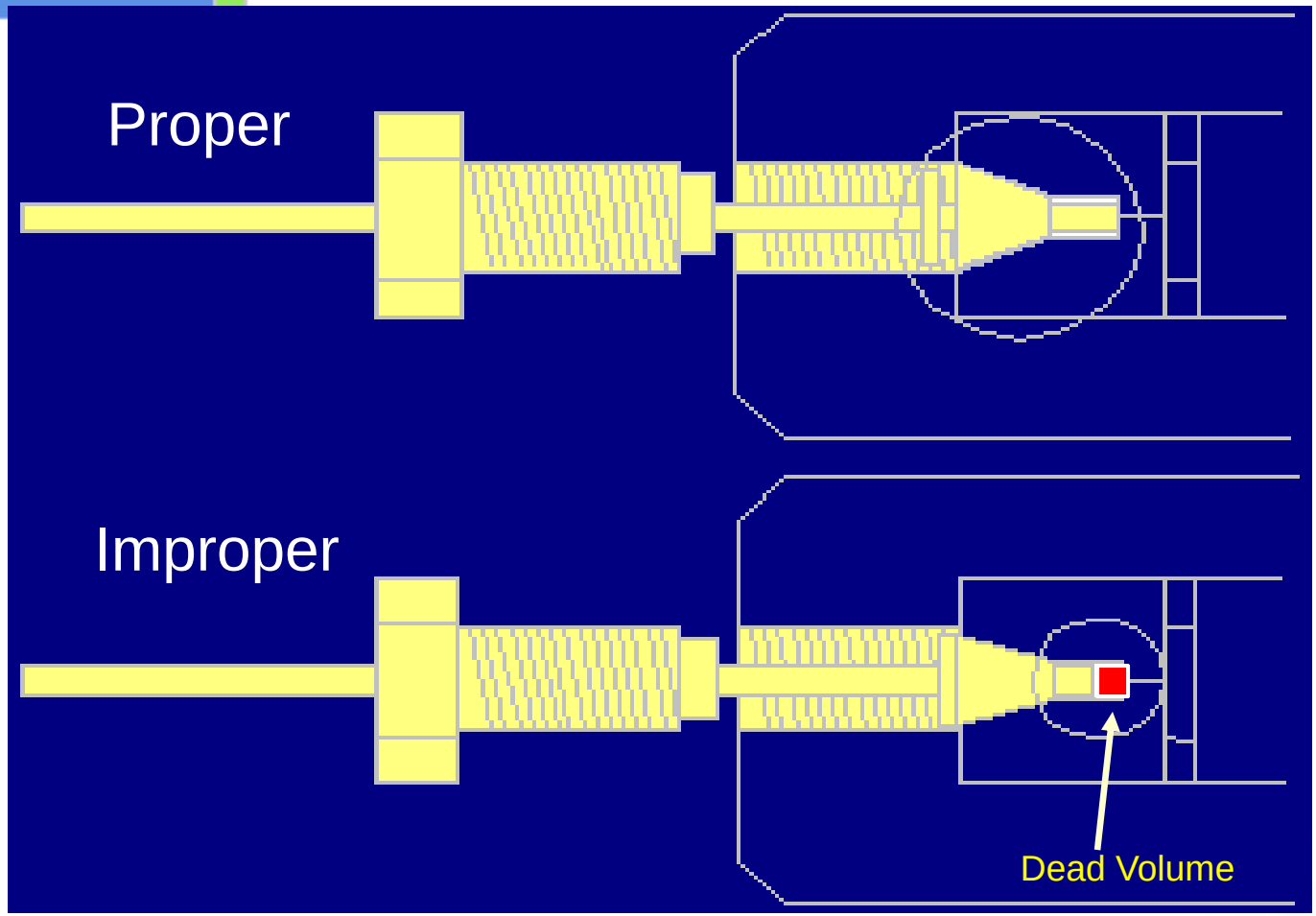
Good Seal

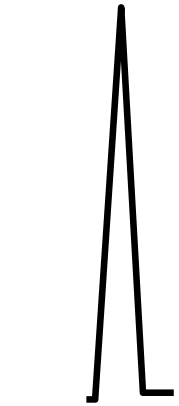
NOTE: Column connector not seated properly -- creates void (Parker ferrule position into a Waters' End-fitting)

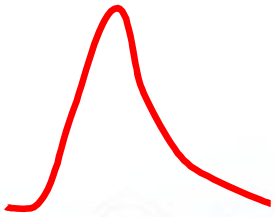


(PEEK Slip Connectors Easier to Use)

Band Spreading due to Improper Column Connection- Mechanical



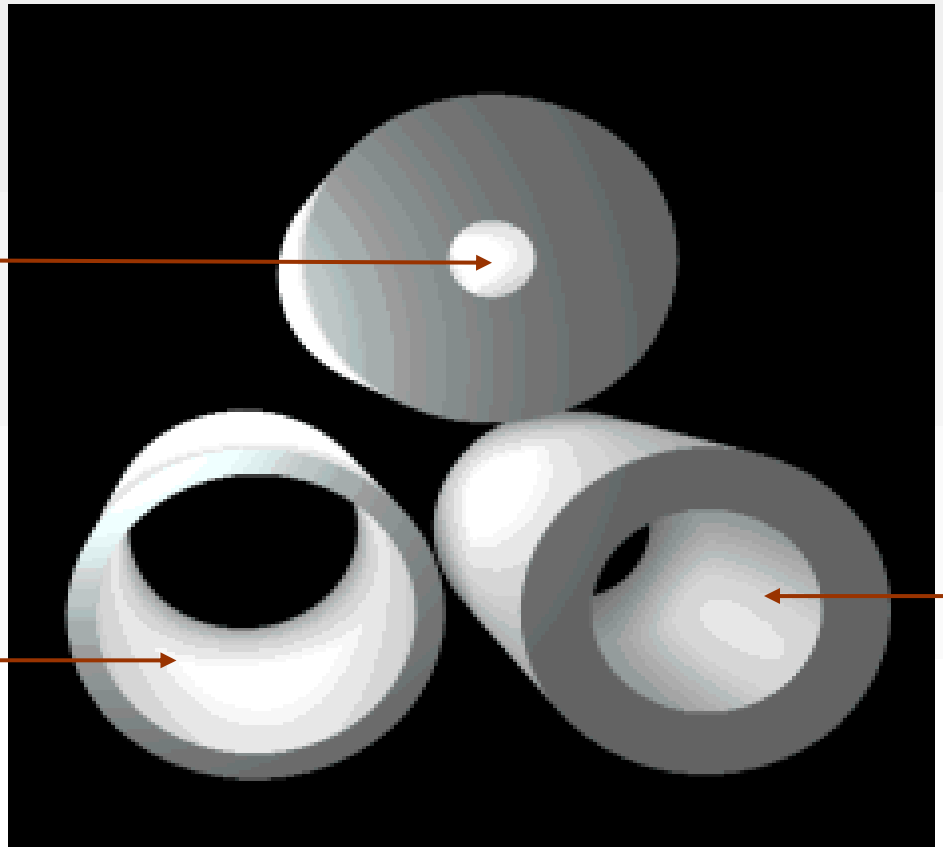




Band Spreading

Column Connection

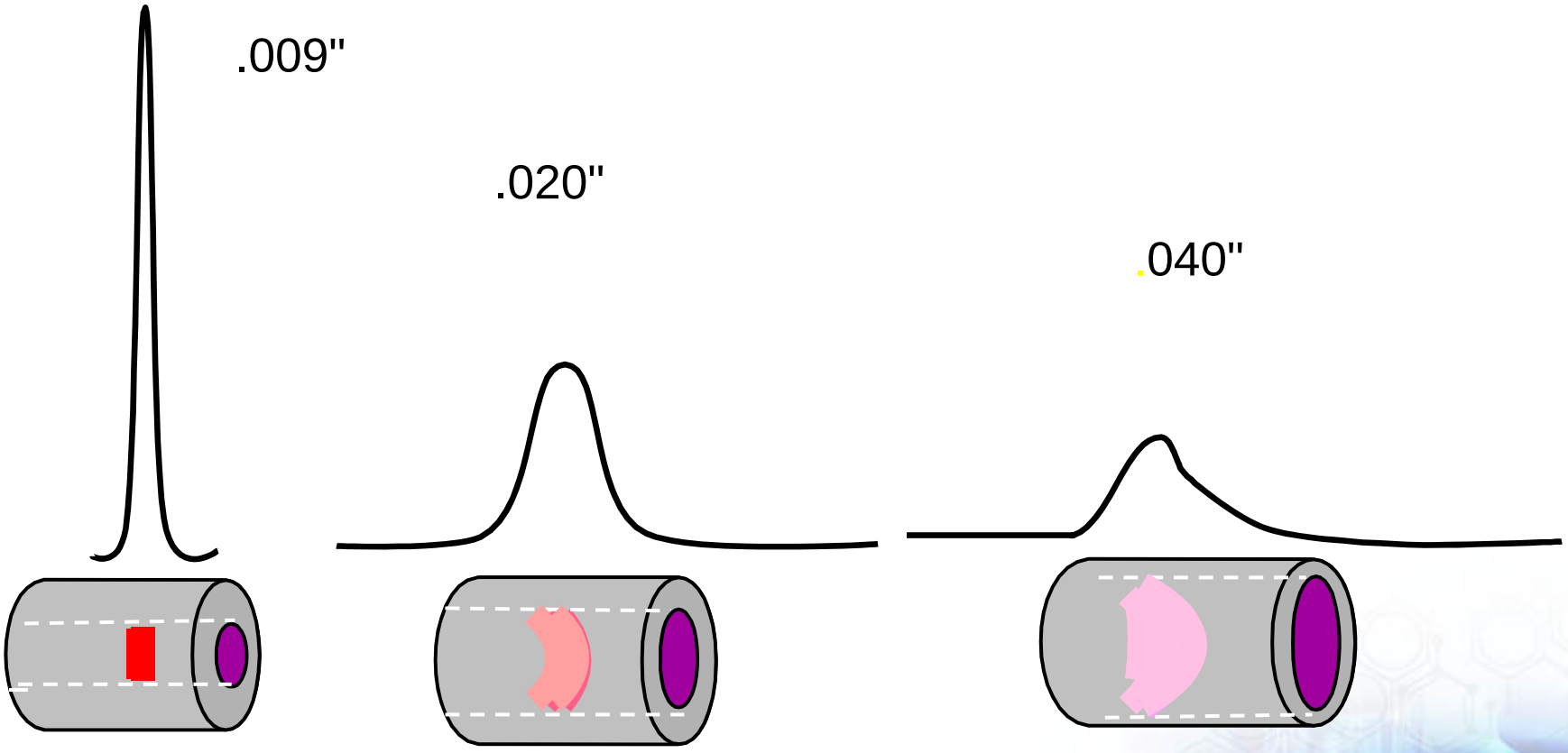
Extra-Column Band Spreading – Tubing ID -- Mechanical



Note the differences for the inner diameter of this connecting tubing

Effect of Connecting Tubing on System Band Spreading

Note: longer tubing lengths creates more Band Spreading

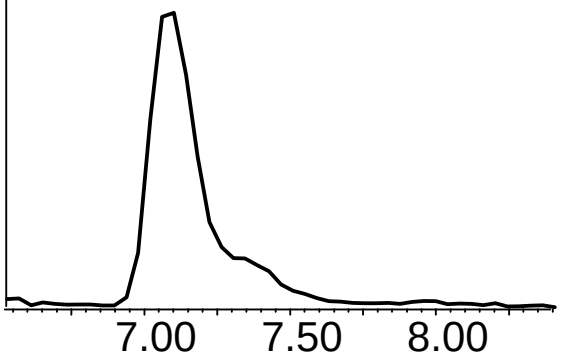


Sample **band** dispersion inside tubing

Effect of Band Spreading on Resolution

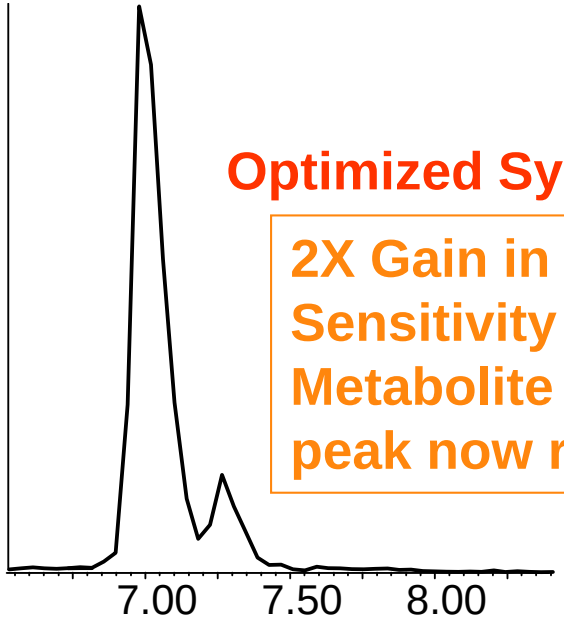
ES- TIC

Original Customer's System



- Customer's plumbing
- Lack of sensitivity
- Quattro Ultima
- Metabolite study

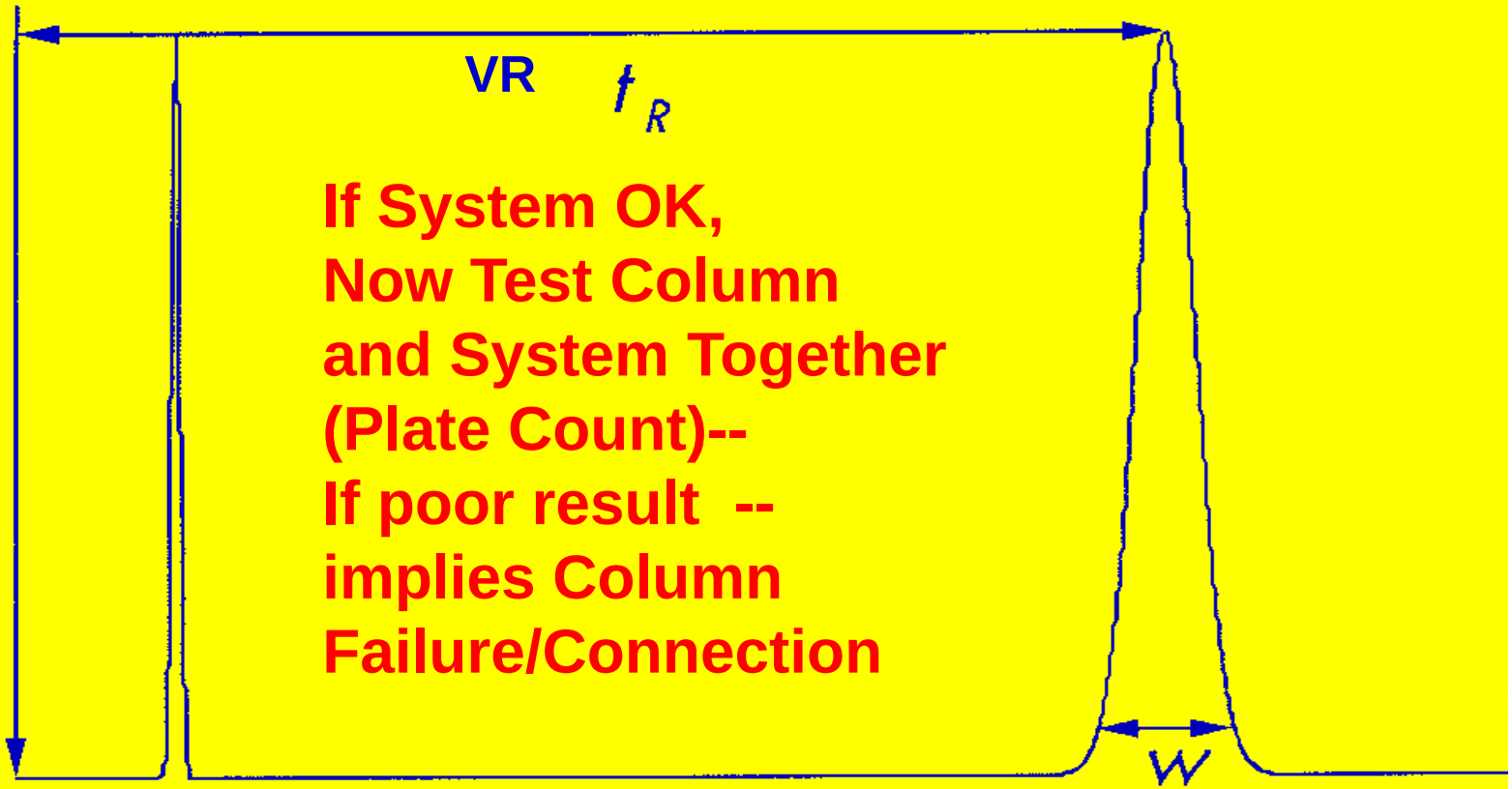
Optimized System



2X Gain in Sensitivity
Metabolite in tail of peak now resolved

- **Improved plumbing**
- **Replace 10 connections between the injector, switching valve and mass spec**
- **Removed 4 feet of extra 0.005 inch id tubing**

Now, Perform Plate Count -- Install Column (Isocratic)



If System OK,
Now Test Column and System Together (Plate Count)--
If poor result -- implies Column Failure/Connection

Plate Count Information included in Appendix

Waters

Performance Monitoring

Column Efficiency:

N = the number of Theoretical Plates

a = is a constant depending on the Method used

t_r = retention time of peak

W = the peak width (time units) at a given peak height

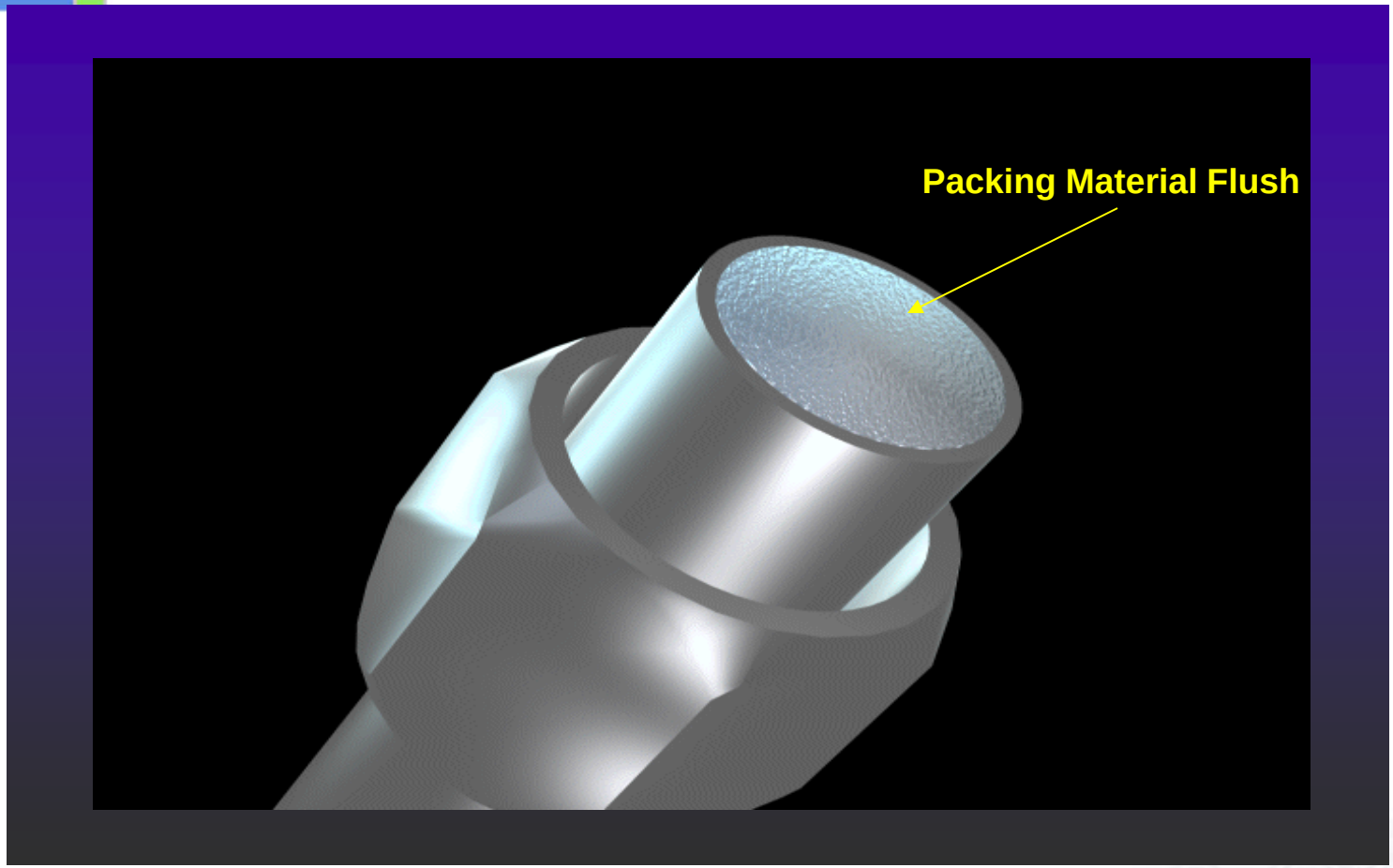
$$N = a \left(\frac{t_r}{W} \right)^2$$

METHOD	a
Peak Width at Half Height	5.54
Peak Width at 4.4% Peak Height (5 Sigma)	25.0
Tangent	16.0

Note: Value will depend on CONDITIONS.
(Analyte k, flow rate, temperature etc.)

Waters

Mechanical -- Column

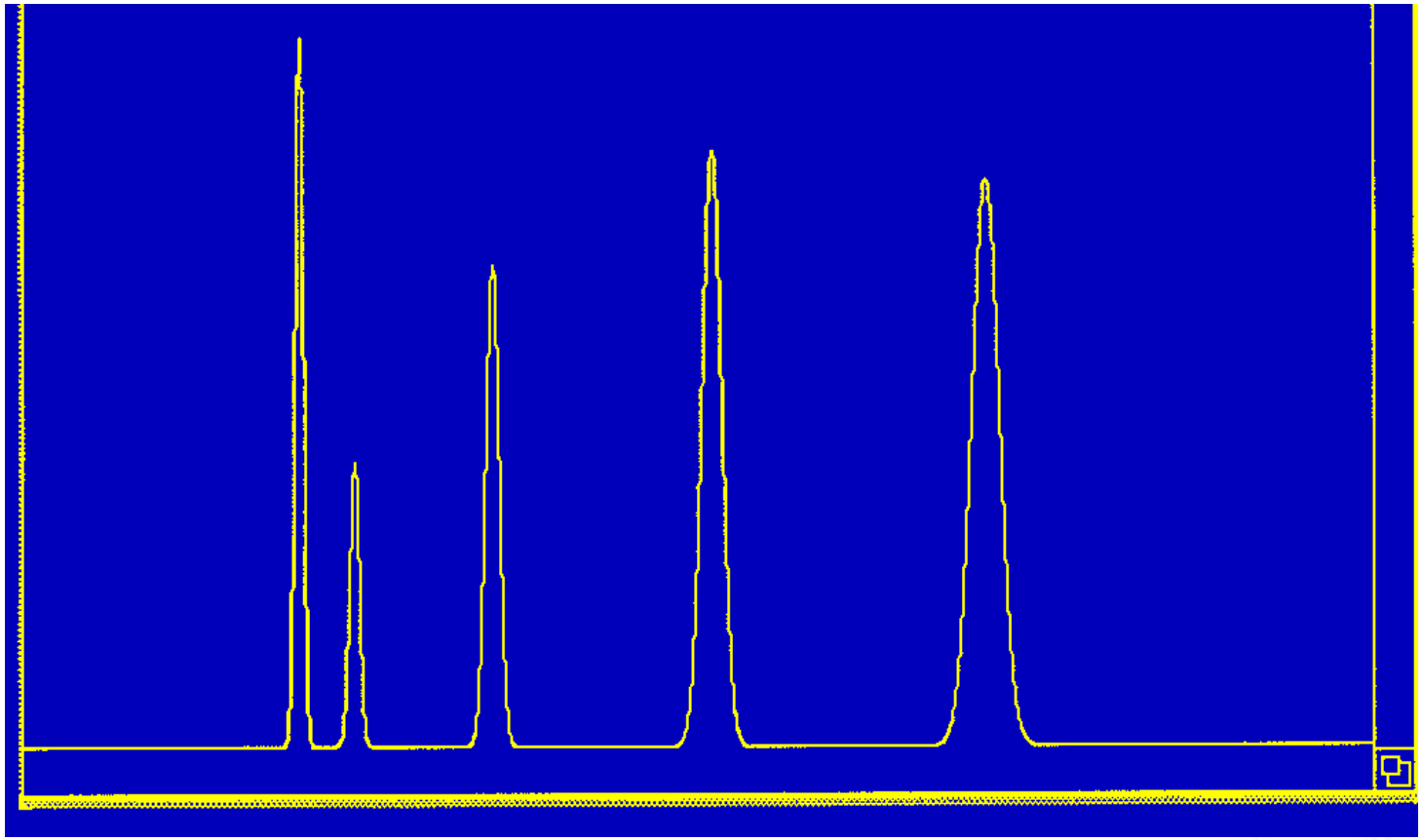


Well packed column

Waters

Good Results

(System OK , Column OK, Connections OK)

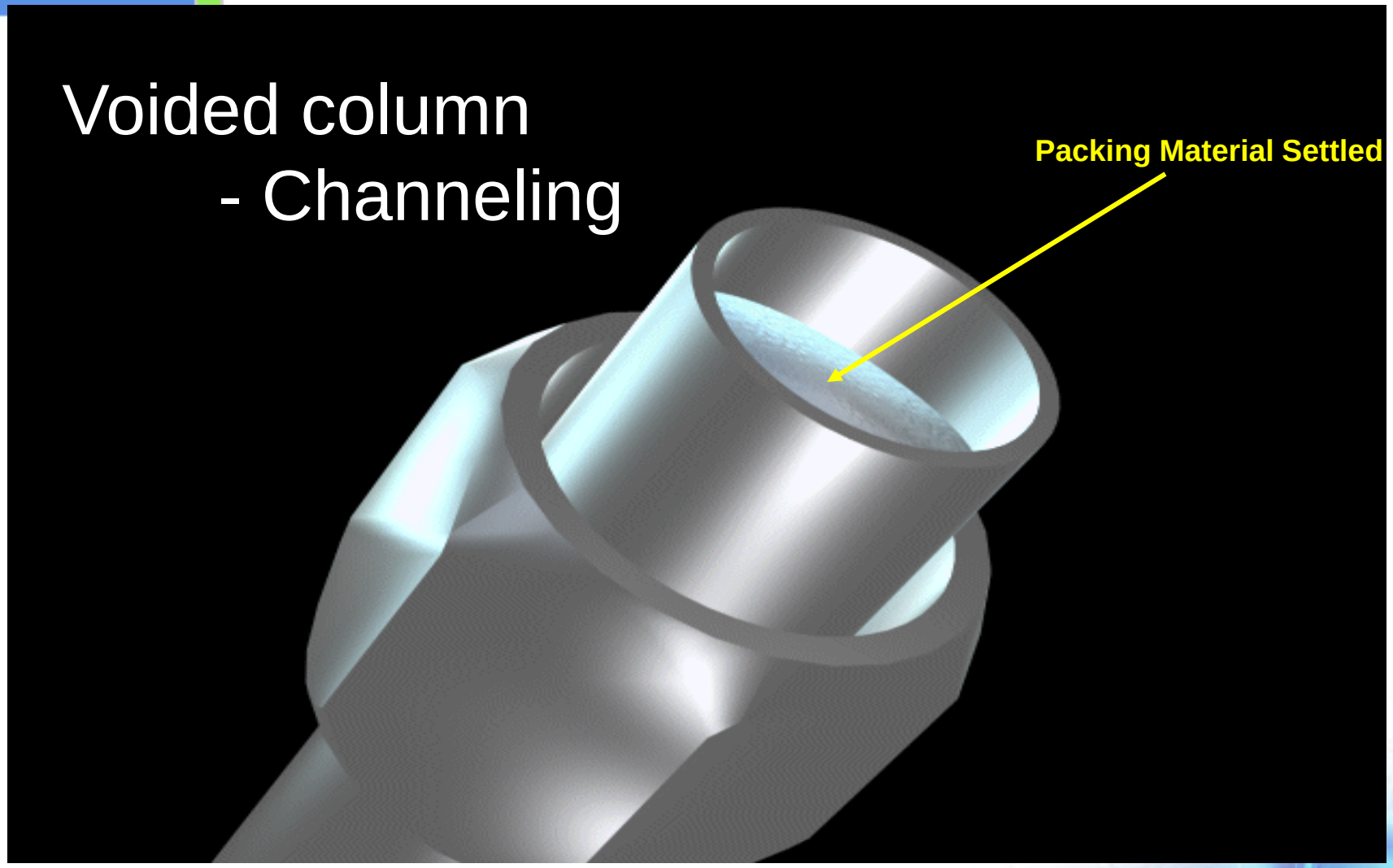


Well packed column

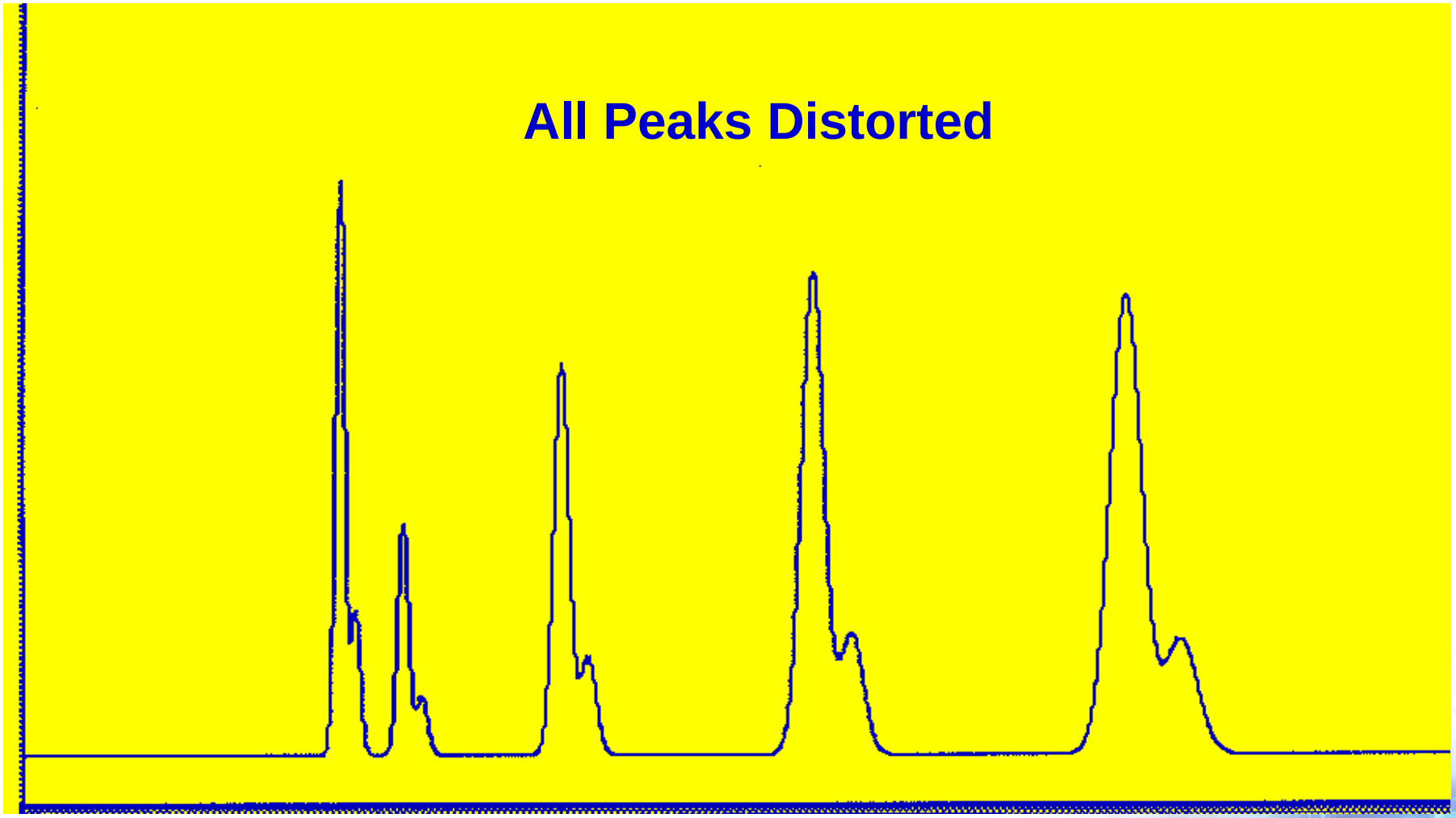
Waters

Column Collapse -- Mechanical

Voided column
- Channeling



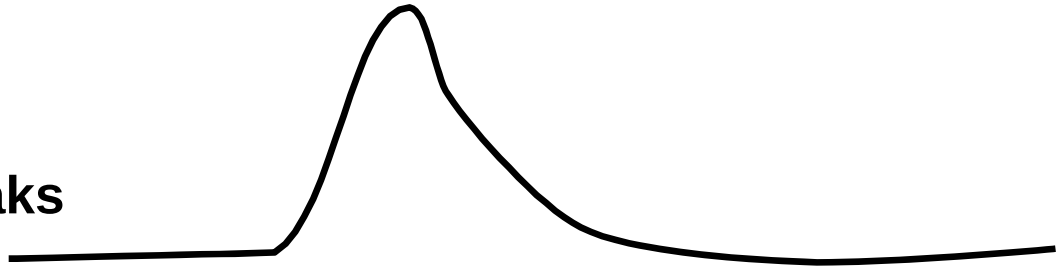
Column Collapse (voiding) (shock / high pH {dissolution of particle})



Other Reasons for Poor Peak Shape

Peak Shape Problems

Broad and Tailing peaks



*** PARTIALLY PLUGGED COLUMN INLET FILTER**
- Remove end-fitting

*** CONTAMINATED IN-LINE FILTER**
- Replace frit

*** CONTAMINATED GUARD COLUMN**
- Replace guard column/insert

See Appendix for more information

Peak Shape Problems

Most Common Problem in HPLC:

- **Distorted peaks will cause integration or resolution problems**

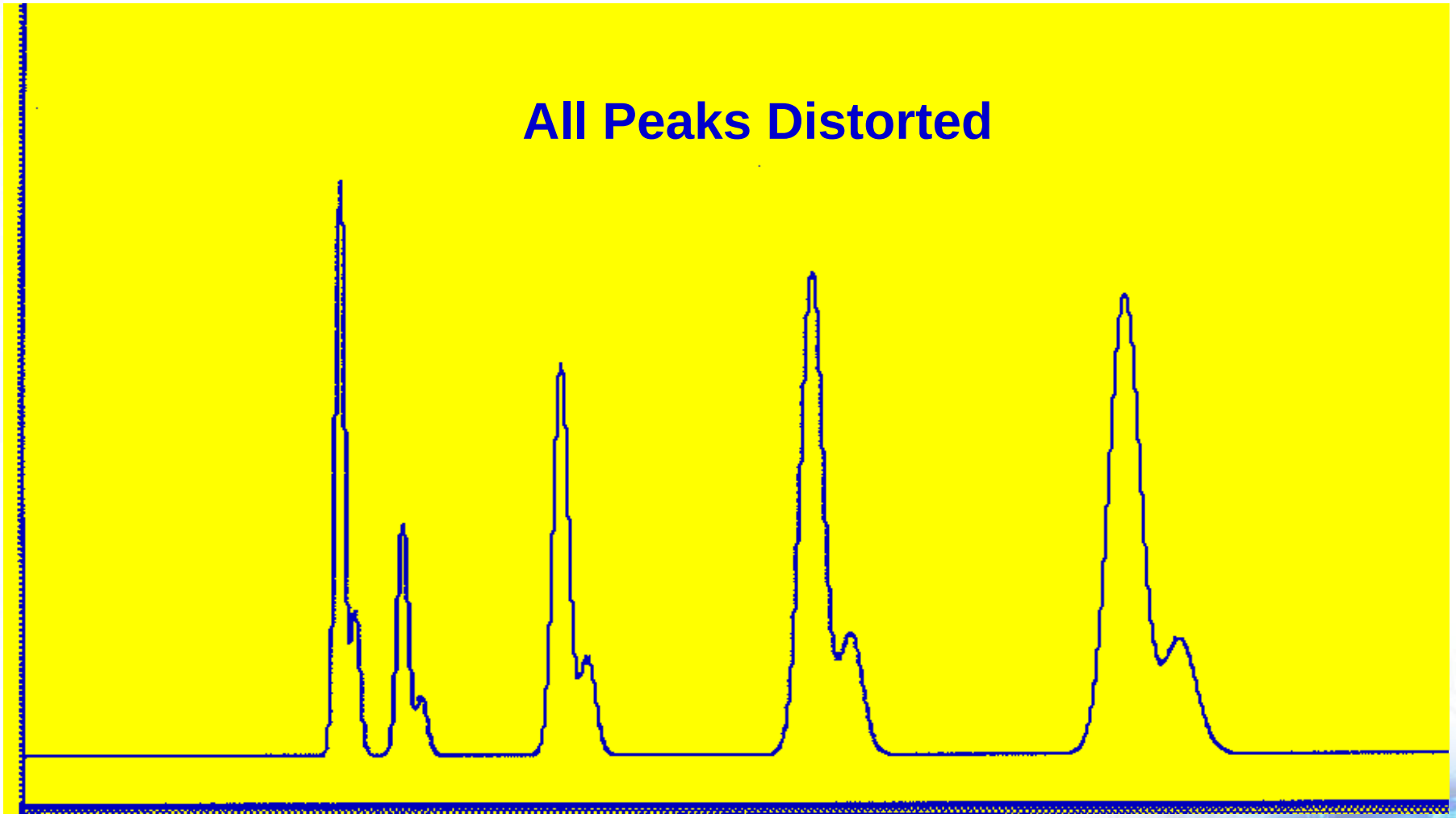
Indication that optimal column performance is not being attained

Waters

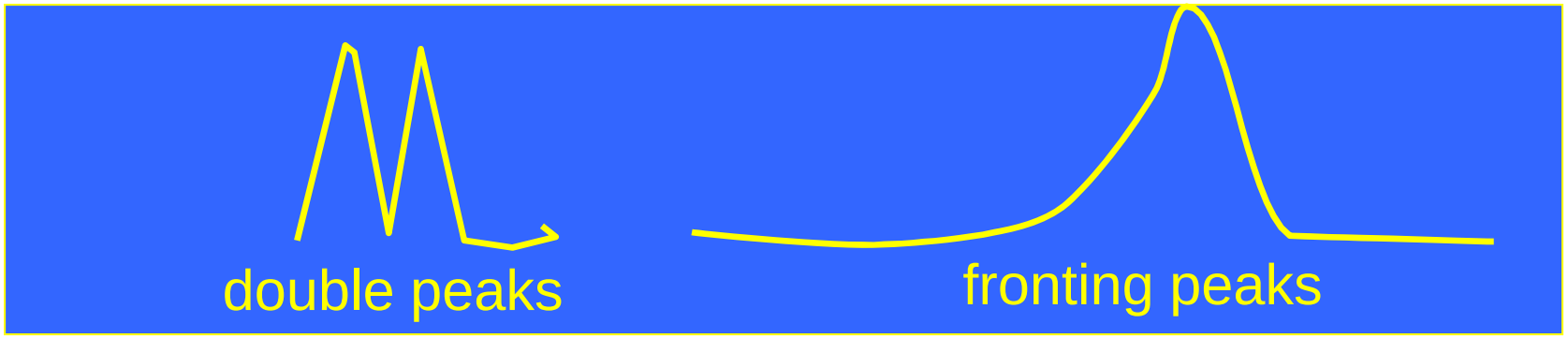
Peak Shape Problems

- Column Destroyed
- Incorrect Sample Solvent
- Secondary Interactions
- Column Overload
  - Mass Overload
  - Volume Overload
- Other Extra-Column Effects
  - Sampling Rate
  - Time Constant

Column Collapse (voiding) (shock / high pH {dissolution of particle})



Peak Shape Problems

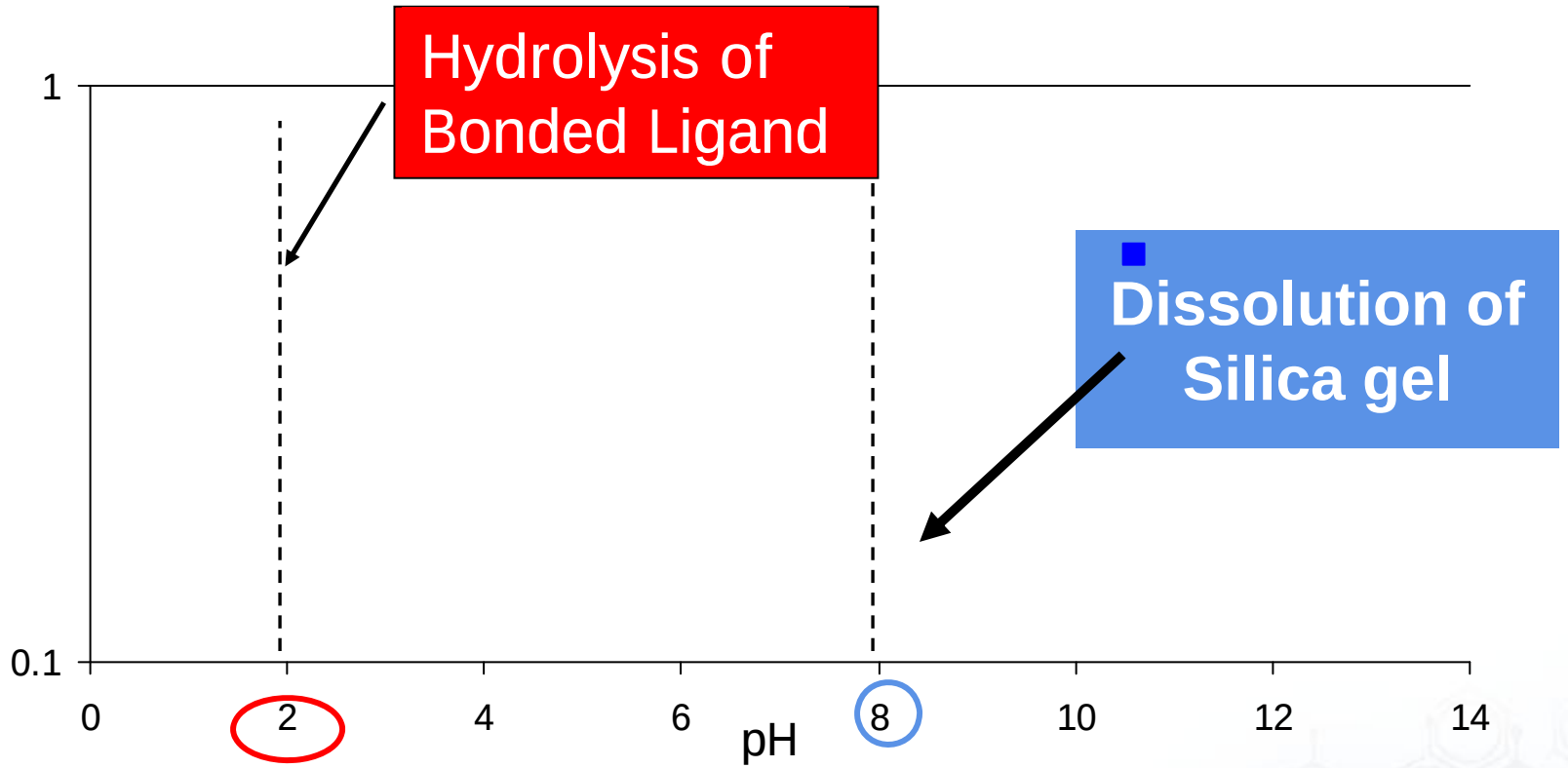


All Peaks Affected

COLUMN
- Connection
- Replace frit/ Guard Column
- Regenerate or replace column

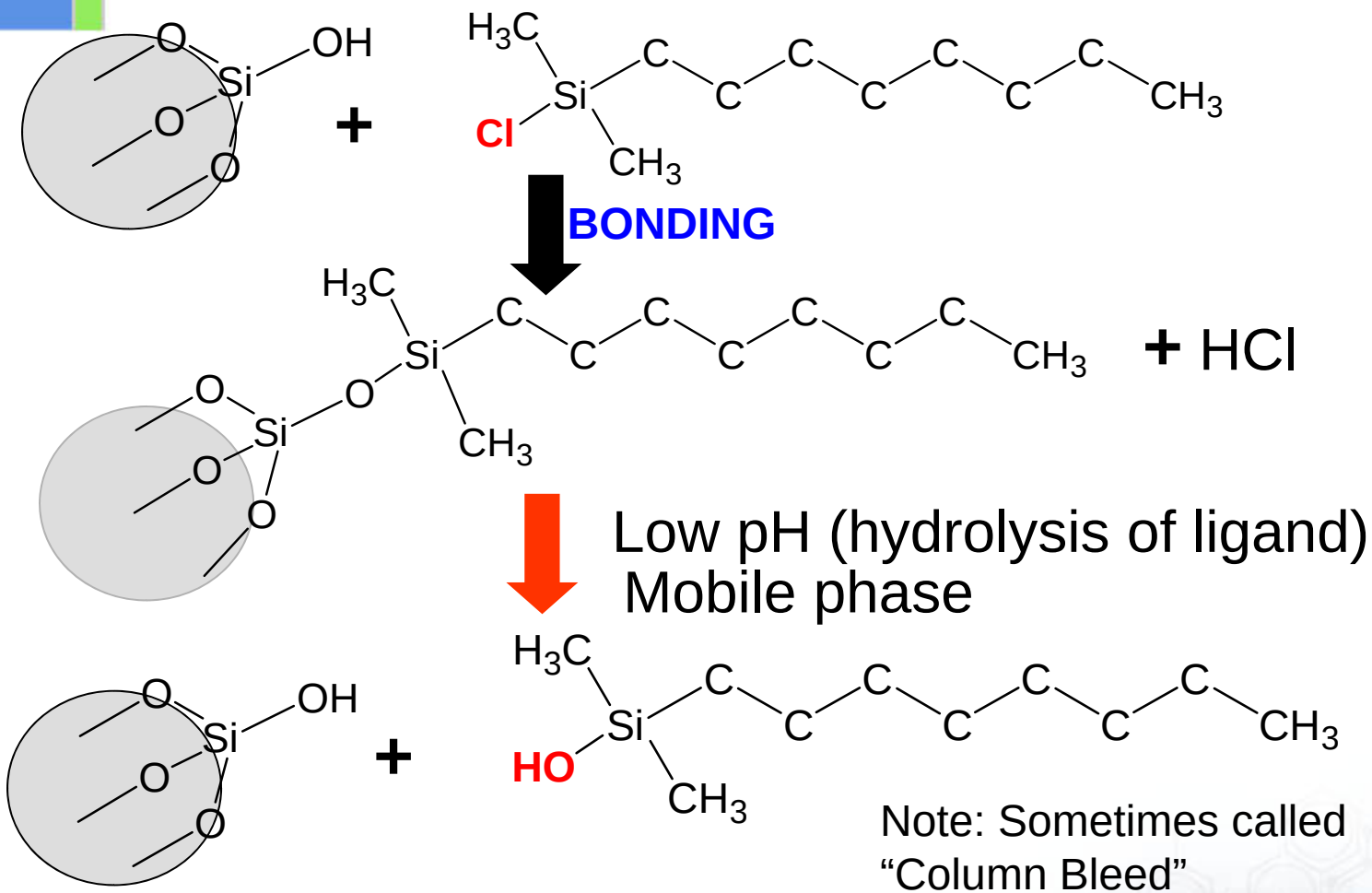
COLUMN ITSELF DESTROYED
pH <2 washes off functional group
pH >8 dissolves silica base

pH Limitations of Traditional Silica Based Packing Materials



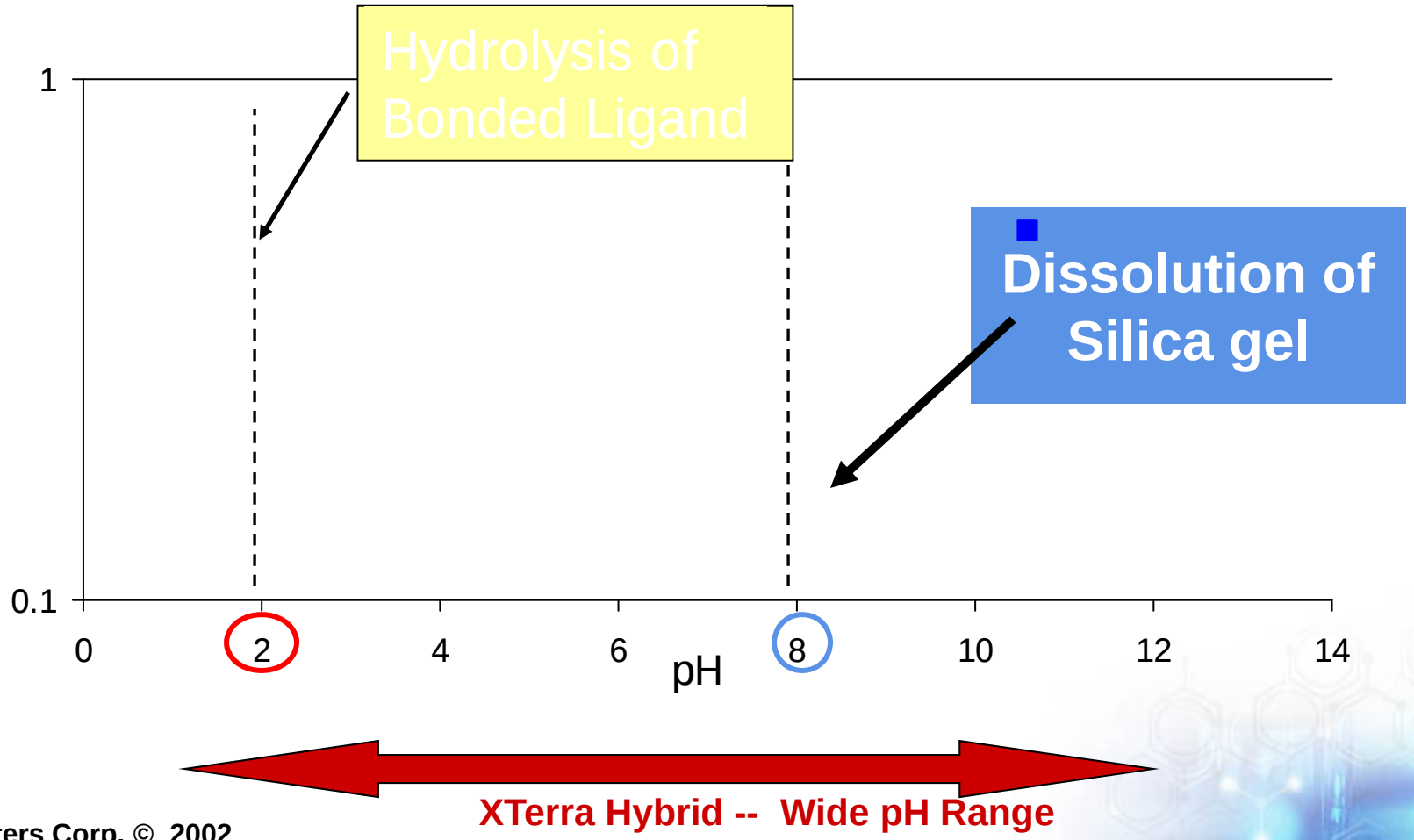
XTerra Hybrid -- Wide pH Range

Hydrolysis of a Bonded Phase Material: Monofunctional Ligand

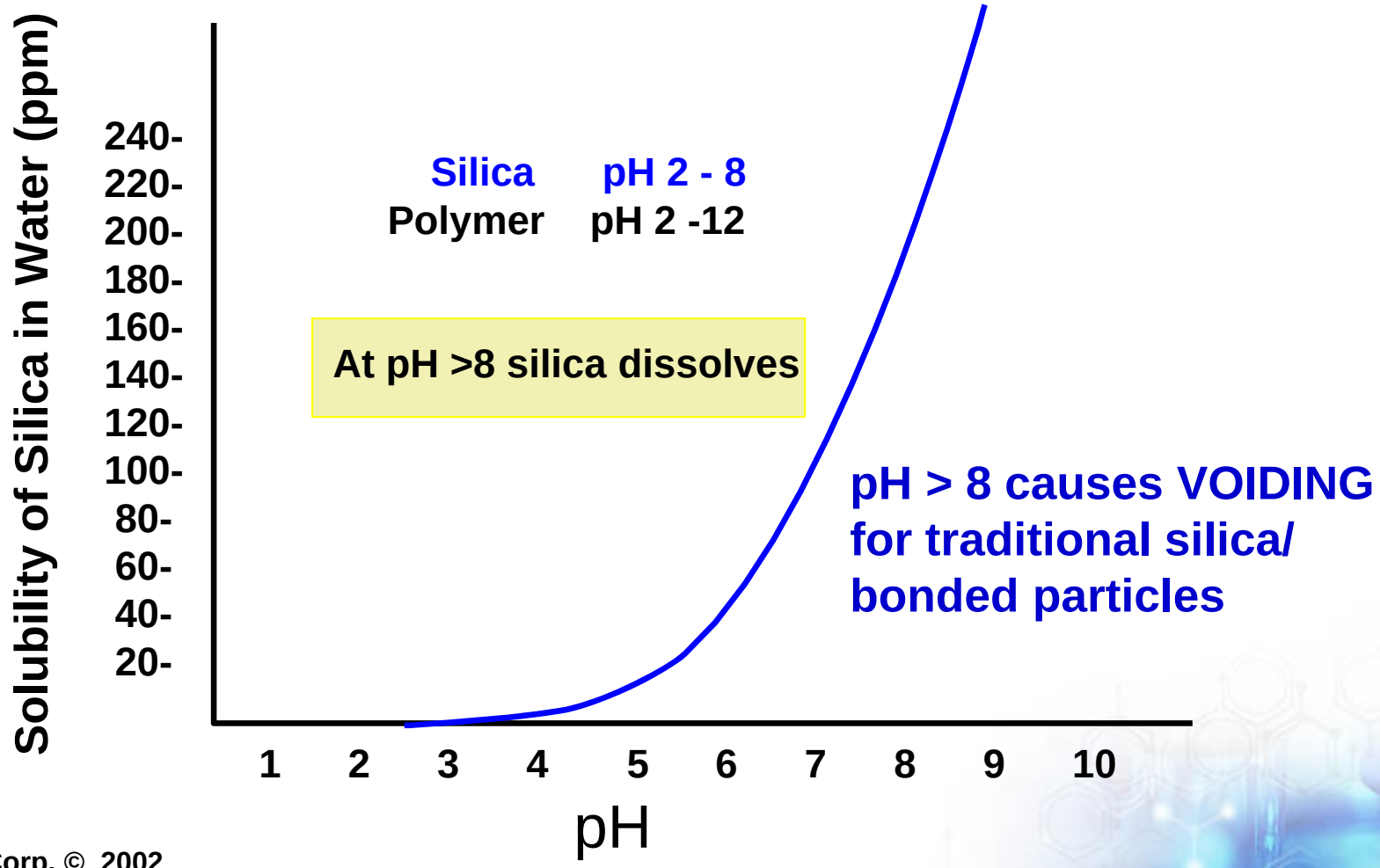


Note: as hydrolysis of ligand continues, you will obtain less retentivity and potentially poor peak shape for bases.

pH Limitations of Traditional Silica Based Packing Materials



Mobile Phase pH and Column Life-time



Waters

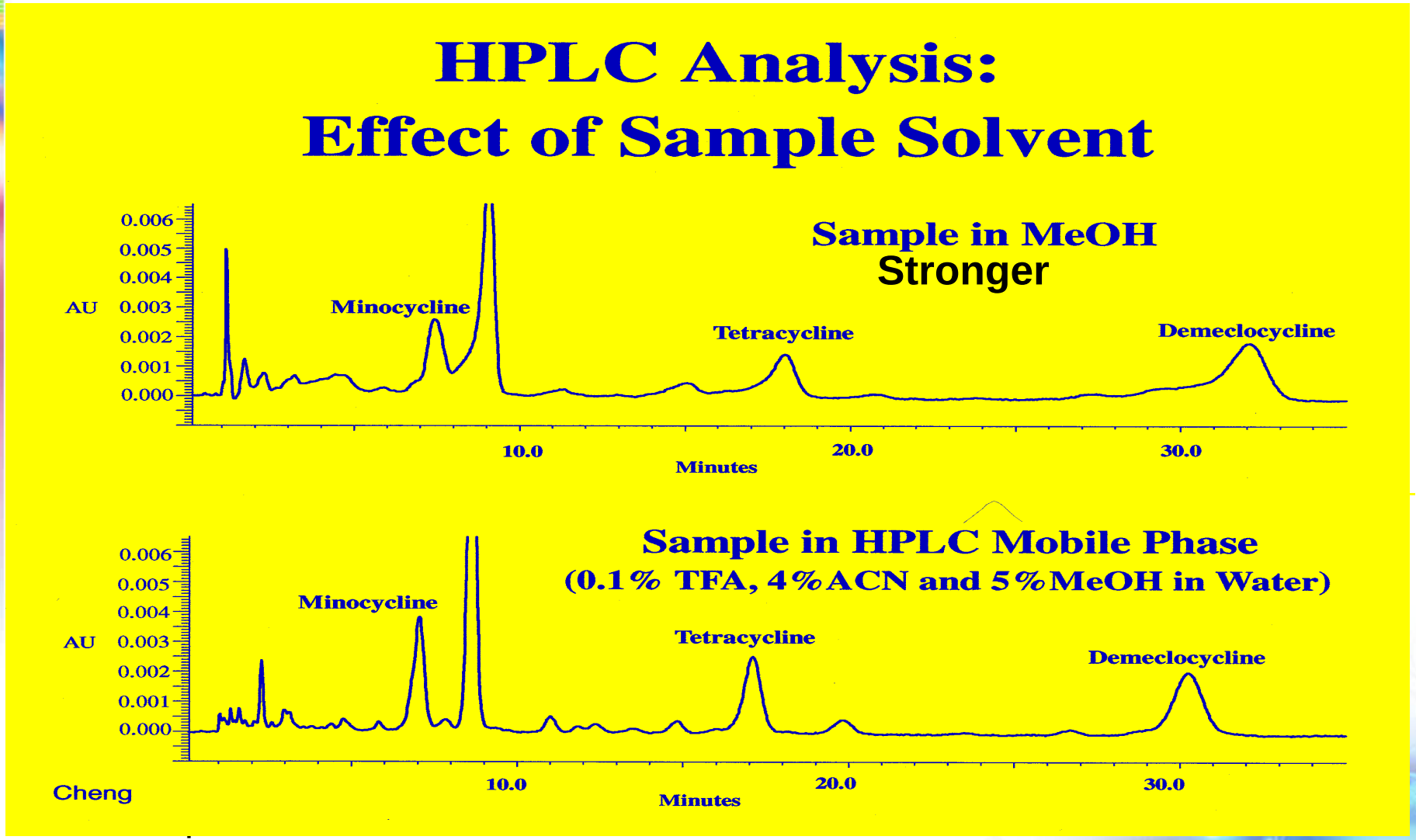
Peak Shape Problems

- Column Destroyed
- Incorrect Sample Solvent
- Secondary Interactions
- Column Overload
  - Mass Overload
  - Volume Overload
- Other Extra-Column Effects
  - Sampling Rate
  - Time Constant

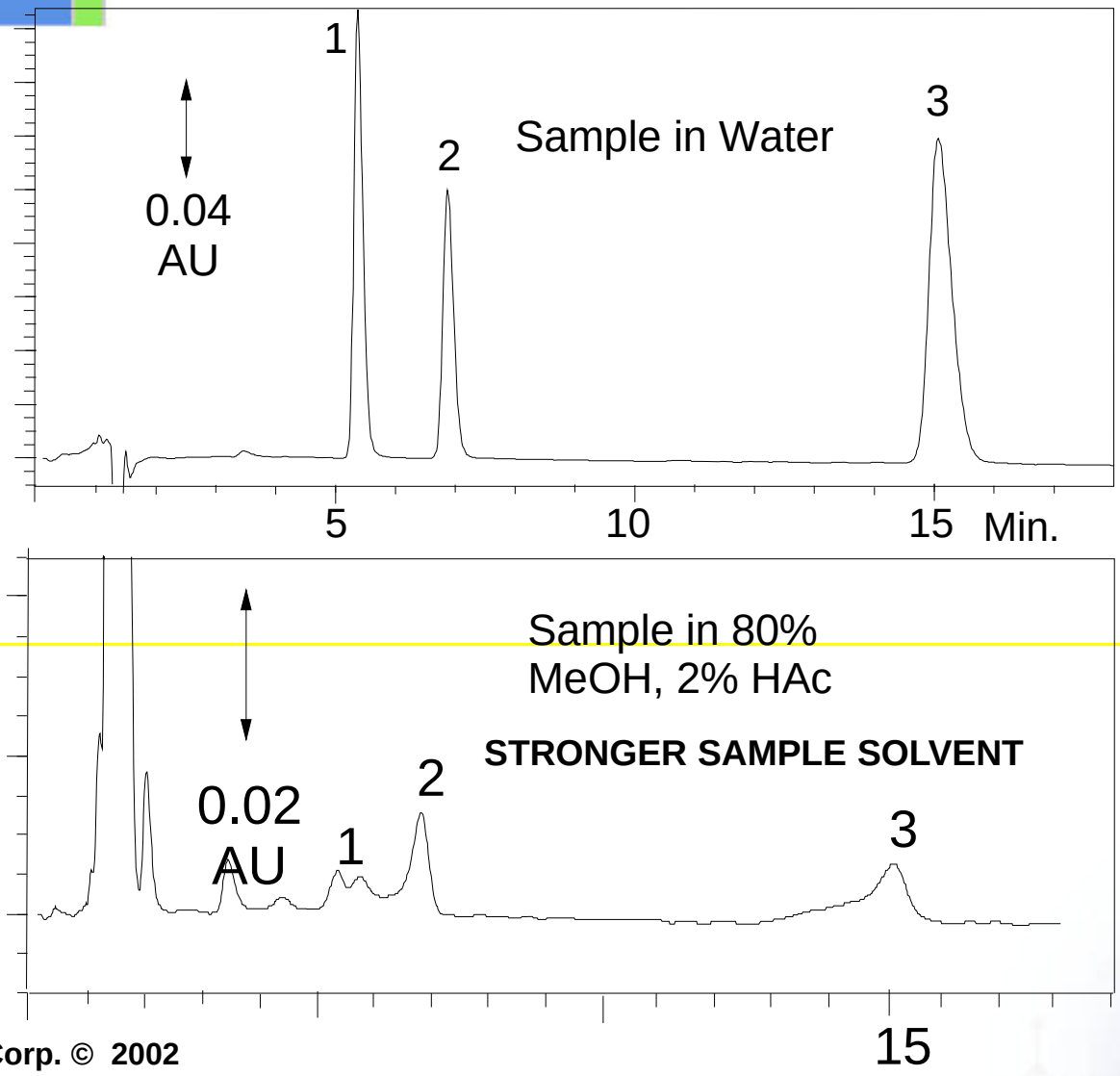
Waters

All Peaks Distorted – Chemical Problem

Incorrect Sample Solvent – STRONGER than mobile phase



All Peaks Distorted – Chemical Problem
Incorrect Sample Solvent – STRONGER than mobile phase



Sample Identification
1.EDDP
2. Diphenhydramine (IS)
3. Methadone

Column:	SymmetryShield™ RP18, 3.5 µm, 3.9 x 150 mm
Guard Column:	Sentry™ Guard Column SymmetryShield RP18, 5µm
Temperature:	30°C
Mobile Phase:	0.1% TFA:Methanol (60:40)
Detection:	UV at 210 nm
Flow Rate:	1 mL/min
Inj. Volume:	30 µL

Waters

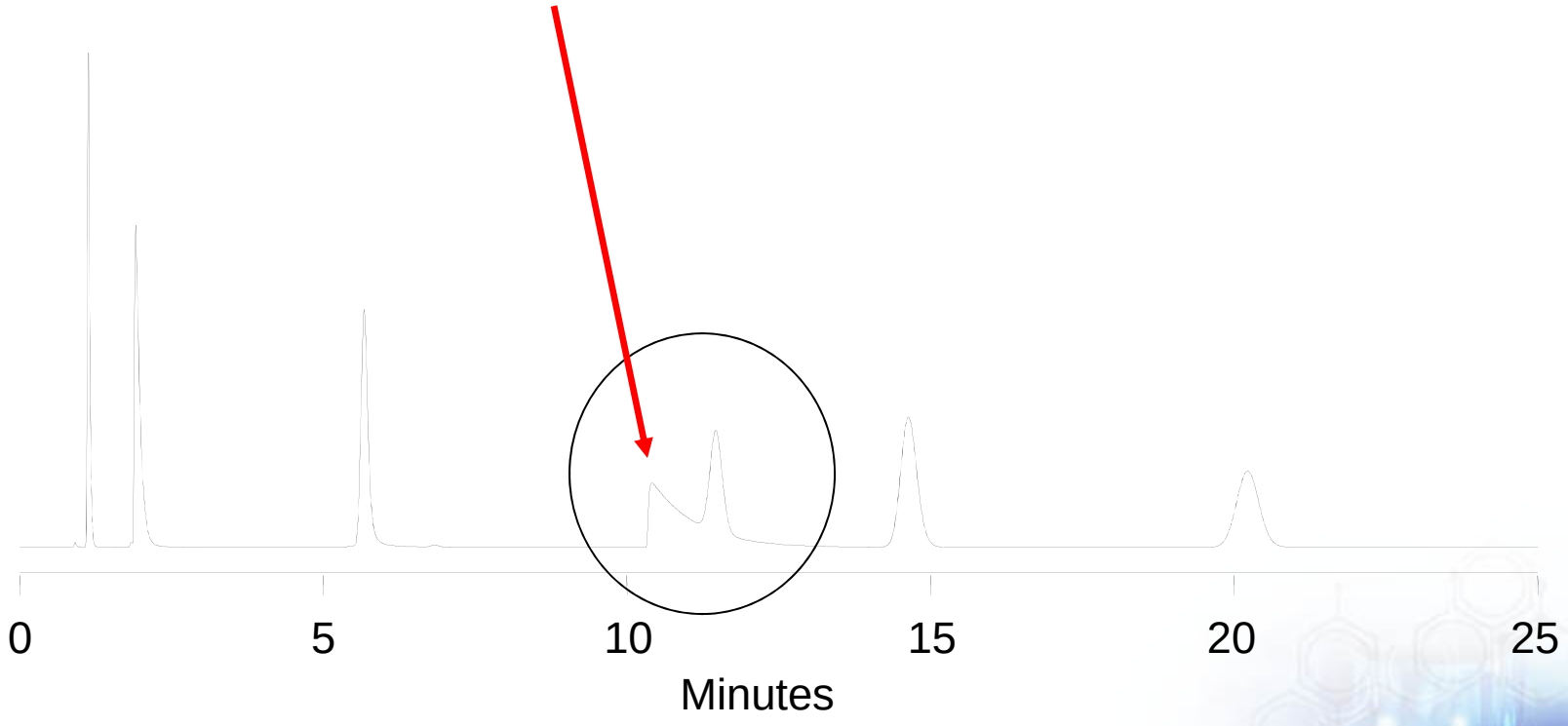
Peak Shape Problems

- Column Destroyed
- Incorrect Sample Solvent
- Secondary Interactions
- Column Overload
  - Mass Overload
  - Volume Overload
- Other Extra-Column Effects
  - Sampling Rate
  - Time Constant

Poor Peak Shape on Basic Compound due to Secondary Interactions

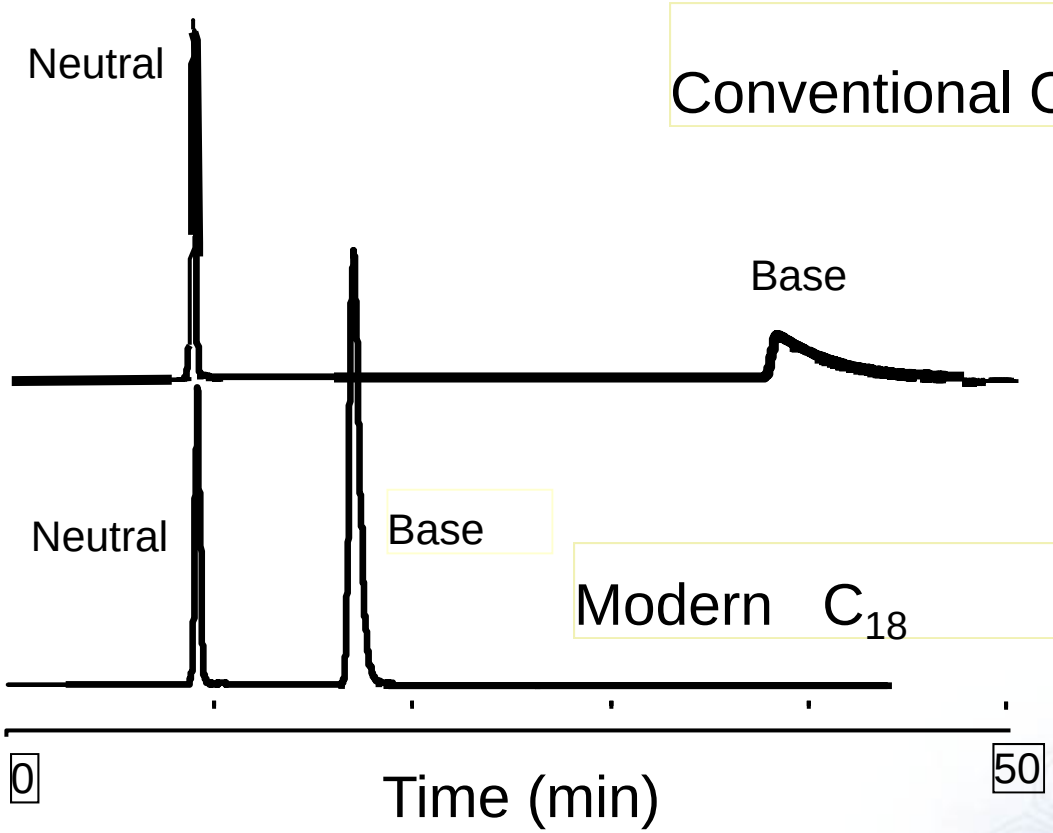
Acids and Neutrals have Good peak shape --
Basic Analyte "Tails"

- Integration Errors
- Reduced Resolution
- Reduced Sensitivity



Tailing of Bases -- Chemical Problem (column brand / pH)

Differences in Silanol Activity of Column Brands



pH 7

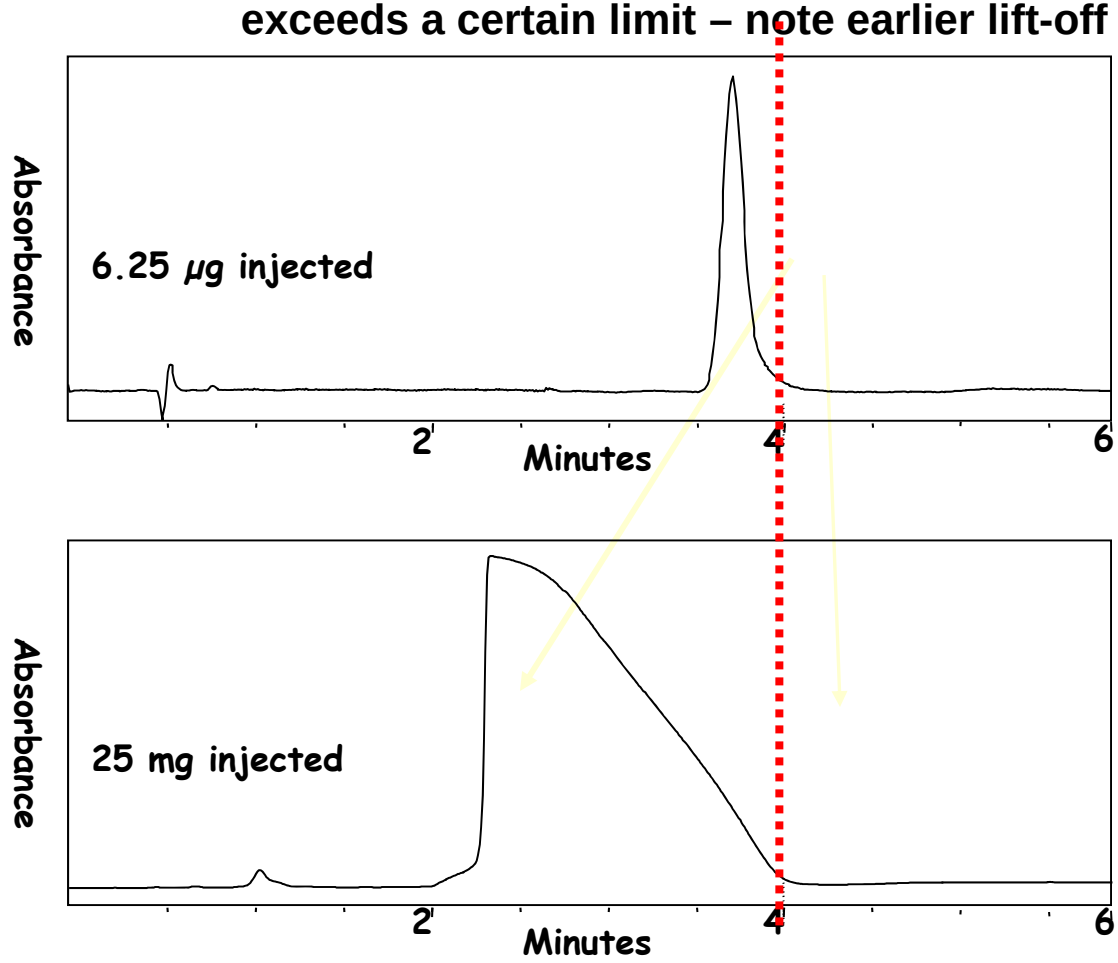
Waters

Peak Shape Problems

- Column Destroyed
- Incorrect Sample Solvent
- Secondary Interactions
- Column Overload
  - Mass Overload
  - Volume Overload
- Other Extra-Column Effects
  - Sampling Rate
  - Time Constant

Concentration/Mass Overload

Encountered when mass injected onto column exceeds a certain limit – note earlier lift-off point

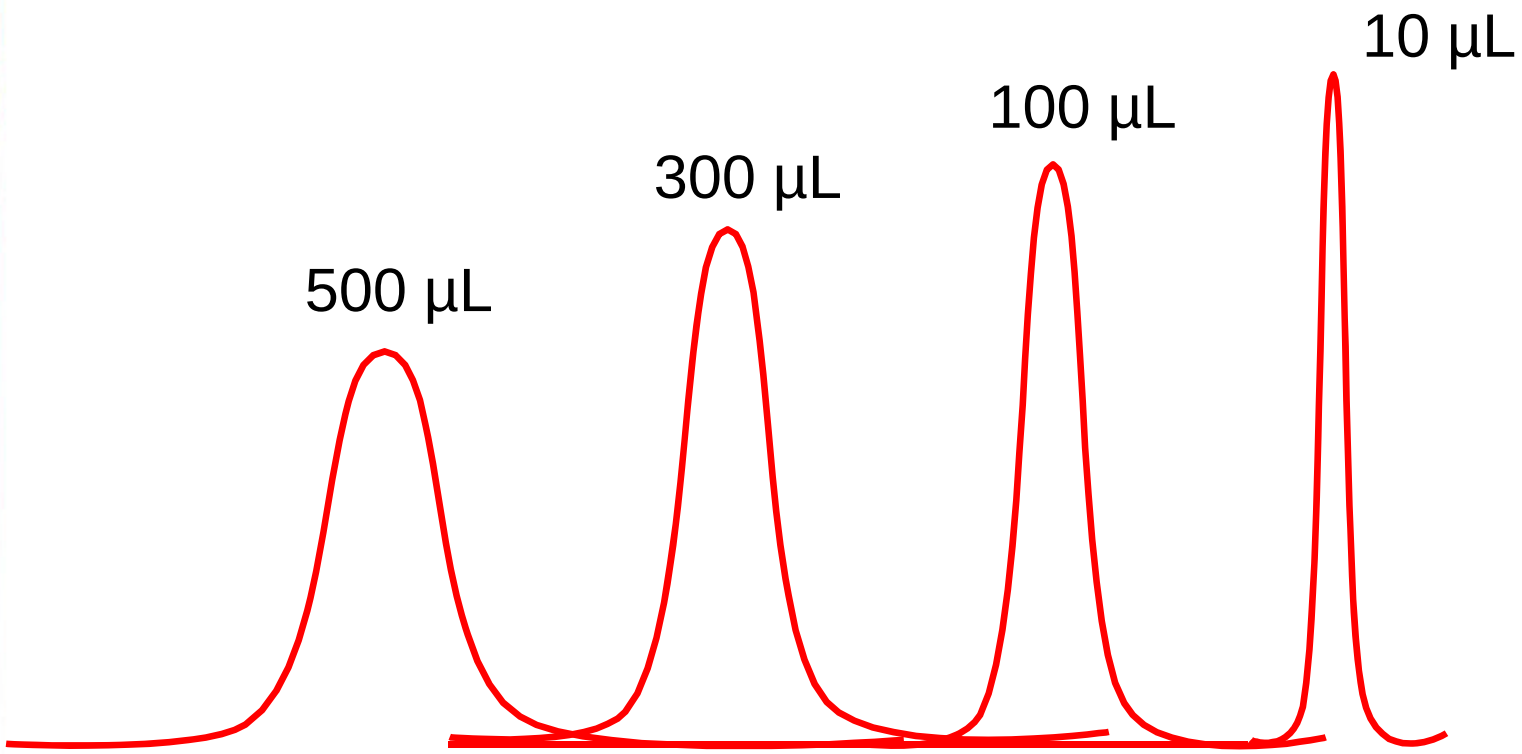


- Analytical load of 6µg yields efficient peak shape
- Preparative load of 25 mg generates mass overload peak shape

- Note that the back of the peaks of the analytical and prep loads are at the same retention (-------)

Waters

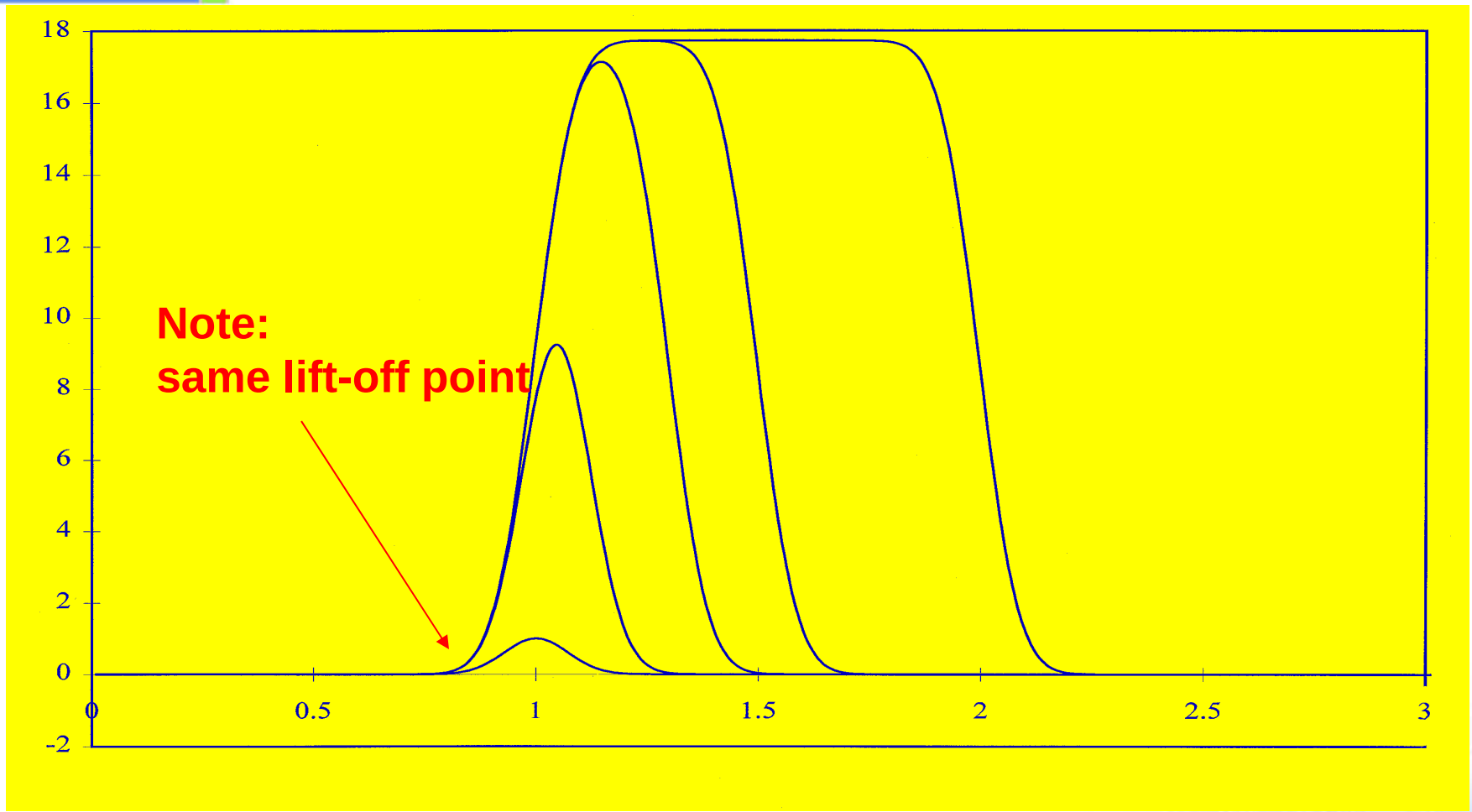
Column/Volume Overload



EFFECT OF INJECTION VOLUME
ON PEAK DISTORTION

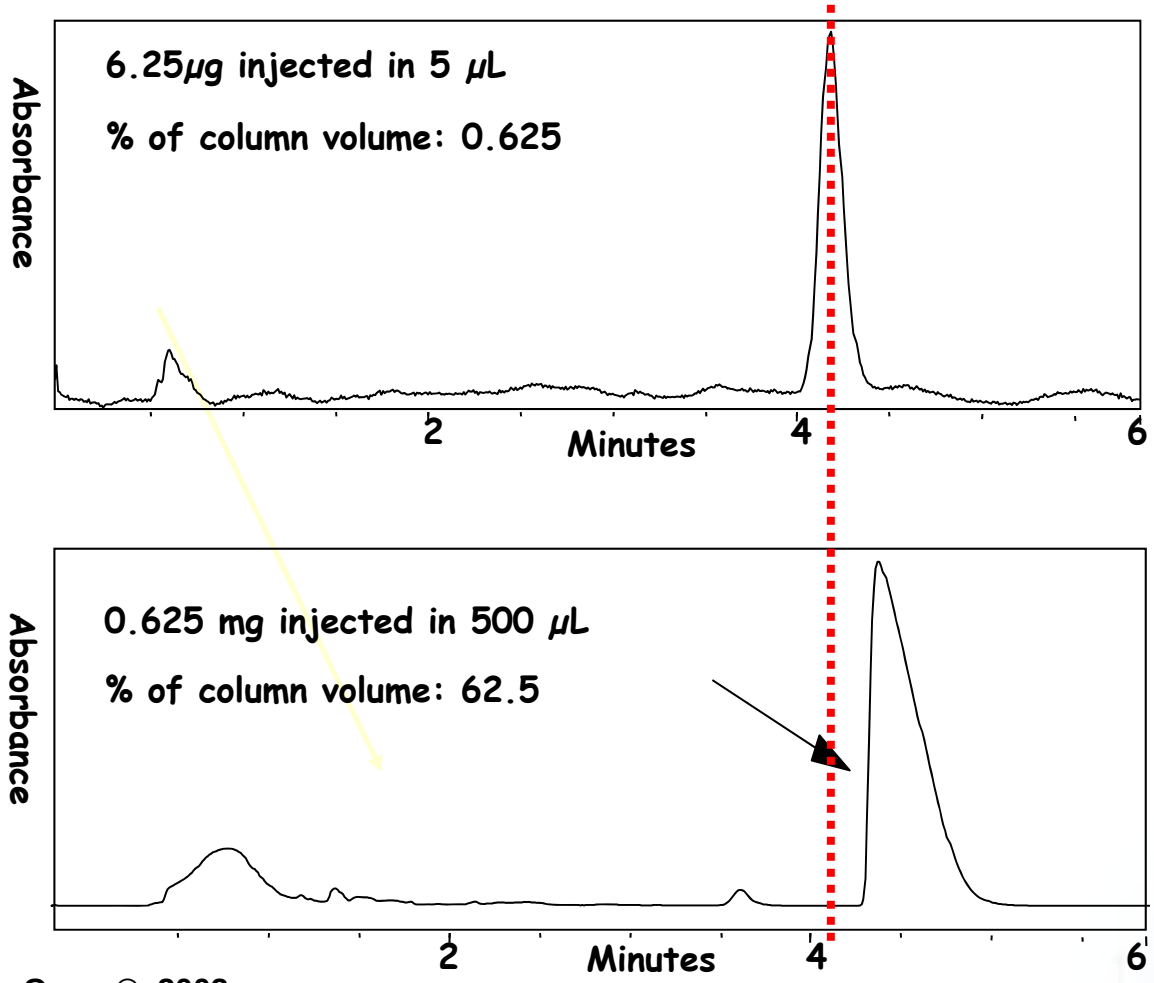
Waters

Volume Overload



Waters

Volume Overload



Column Volume:
0.8 mL (800 µL)

Wider peaks first observed at low retention

Peak position shifts to higher retention in proportion to the injection volume

Waters

Peak Shape Problems

- Column Destroyed
- Incorrect Sample Solvent
- Secondary Interactions
- Column Overload
  - Mass Overload
  - Volume Overload
- Other Extra-Column Effects
  - Sampling Rate
  - Time Constant

Course Outline

Part 2 of 3

- **Retention Time Reproducibility**
  - -Temperature
  - - Organic %
  - - pH
  - - Ion Pairing
  - - Hydrophobic Collapse

Methods Development Suggestions

Extraneous Peaks

Retention Time Problems

- **Reproducibility** &

  - Solvent Composition
  - Temperature
  - pH-Control
  - Ion Pairing

Drifting Retention

- Equilibration
- Stationary Phase Stability
- Column Contamination
- Hydrophobic Collapse
  - Low organic < 5%

Retention Time Variability

Less or More Retention Time
All Peaks

- **Pump Flow Rate Problem (check actual volume/time being delivered)**
- **Wrong Column Type (C8 – less retention, vs C18 – more retention)**
- **Temperature Problem (warmer – less retention, colder – more retention)**
- **% Organic In Mobile Phase (more organic – less retention, less organic – more retention)**

Waters

Retention Time Reproducibility

Non-Column Influences:

Temperature

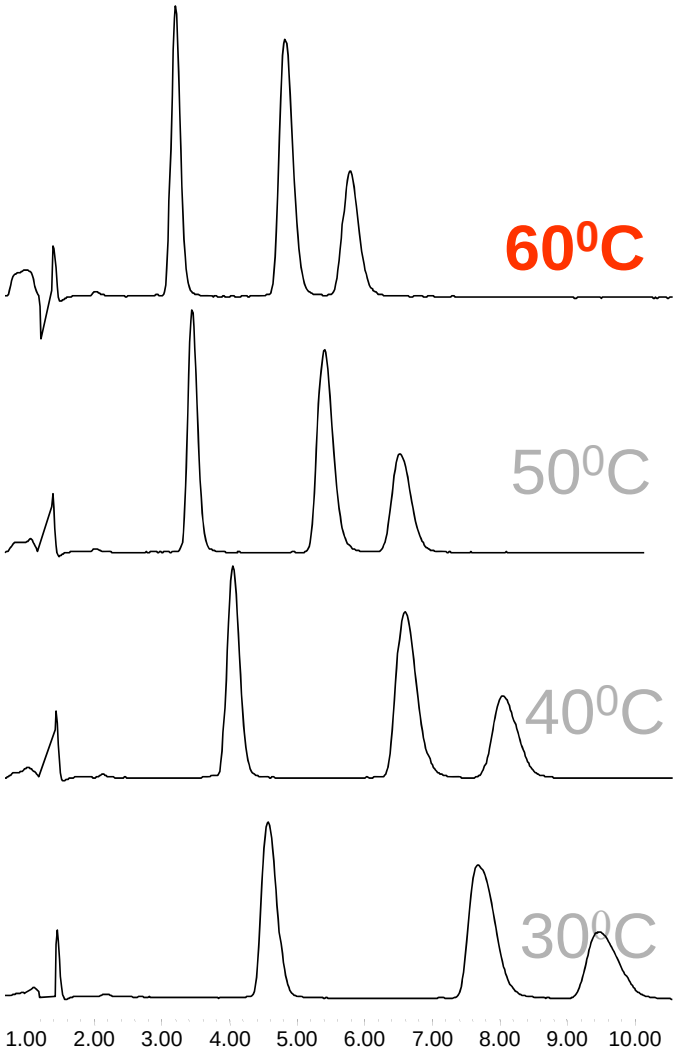
- Reduction of Retention with Increasing Temperature
- 1% to 2% Change / per 1° Celsius
- Shifts in Selectivity (Usually Small)

Effect of Temperature (Isocratic Separations)

Plates

N=2250

N= 1680



Back Pressure

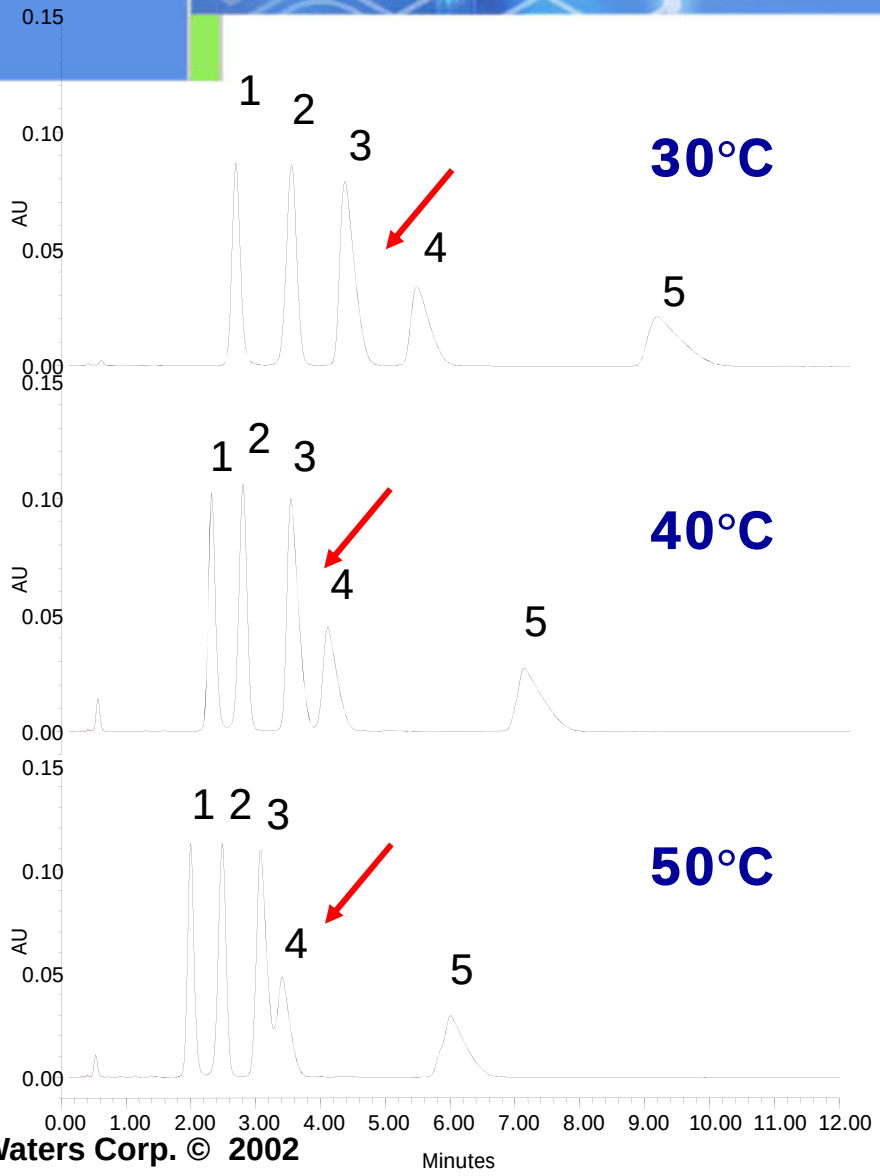
1160 psi

1920 psi

Benefits of Higher Temperature:

* Shorter Run Time
* Better Sensitivity
* Sharper Peaks, N
* Lower Back Pressure (less viscosity)

Temperature



Small, but significant selectivity changes obtained from temperature changes.

Useful for selectivity fine tuning.

1 Triamterene
2 Althiazide
3 Bumetanide
4 Benzthiazide
5 Ethacrynic Acid

Column: XTerra MS C_{18}
3.5 µm, 4.6 mm x 50 mm
Mobile phase:
25% MeOH, 65% water, 10% ammonium bicarbonate buffer, pH 9

Wagrowski, Tran

Waters

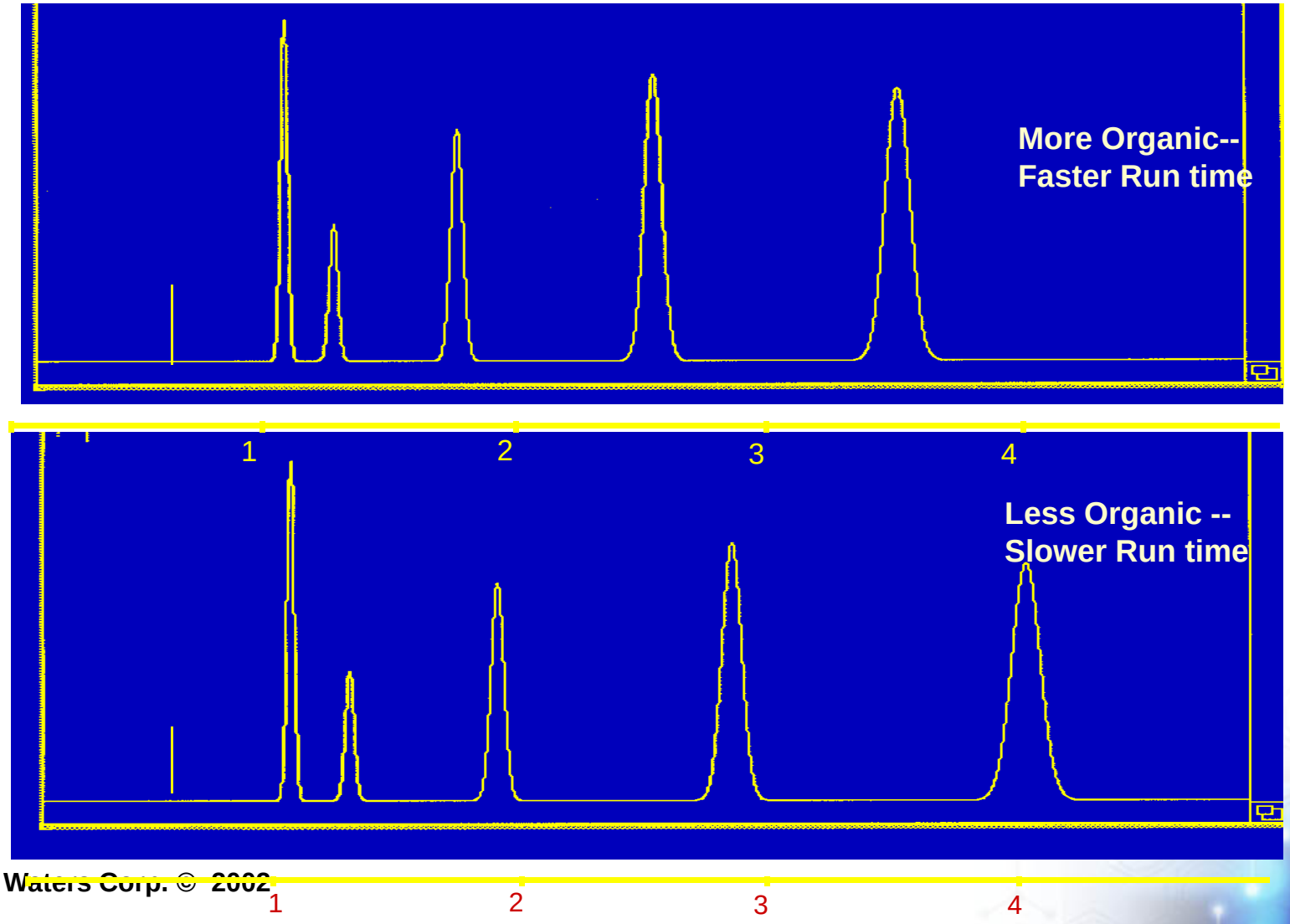
Retention Time Reproducibility

Non-Column Influences:

Solvent (Organic) Composition

- Exponential Relationship between k and Volume %
- Retention Time Change of 5% to 15% per 1% Change in Solvent Composition
- Bonded Phase Collapse in High Water Content

Isocratic LC - Changing Retention Time Change in Solvent Composition



Retention Time Variability

Less or More Retention Time

Some Peaks

- **Chemistry Problem**
  - **Wrong Column Type (CN vs C18)**
  - **Incorrect pH or un-buffered system**
  - **Incorrect % Organic In Mobile Phase, or wrong organic solvent**

Retention Time Reproducibility

Non-Column Influences:

Ion Pairing Reagents (Hexane Sulfonic Acid)

- Retention
  - Increases Proportional to the Concentration of the Pairing Agent at Low Concentration
  - Nearly Independent of the Concentration of the Pairing Agent at High (~10mM/L) Concentration

- Long Equilibration Times
  - Due to Adsorption of the Reagent on the Stationary Phase (can be up to 500 Column Volumes)

Equilibration of Ion Pairing Methods

- Columns using Paired Ion Mobile Phase
  - require significantly more equilibration time

1 µmol/m^2	300 m^2/g	2 g
(Surface coverage)	(Silica surface area)	(Amt. material in column)

Equals 600 µmoles of ion pairing reagent needed for surface coverage in this column

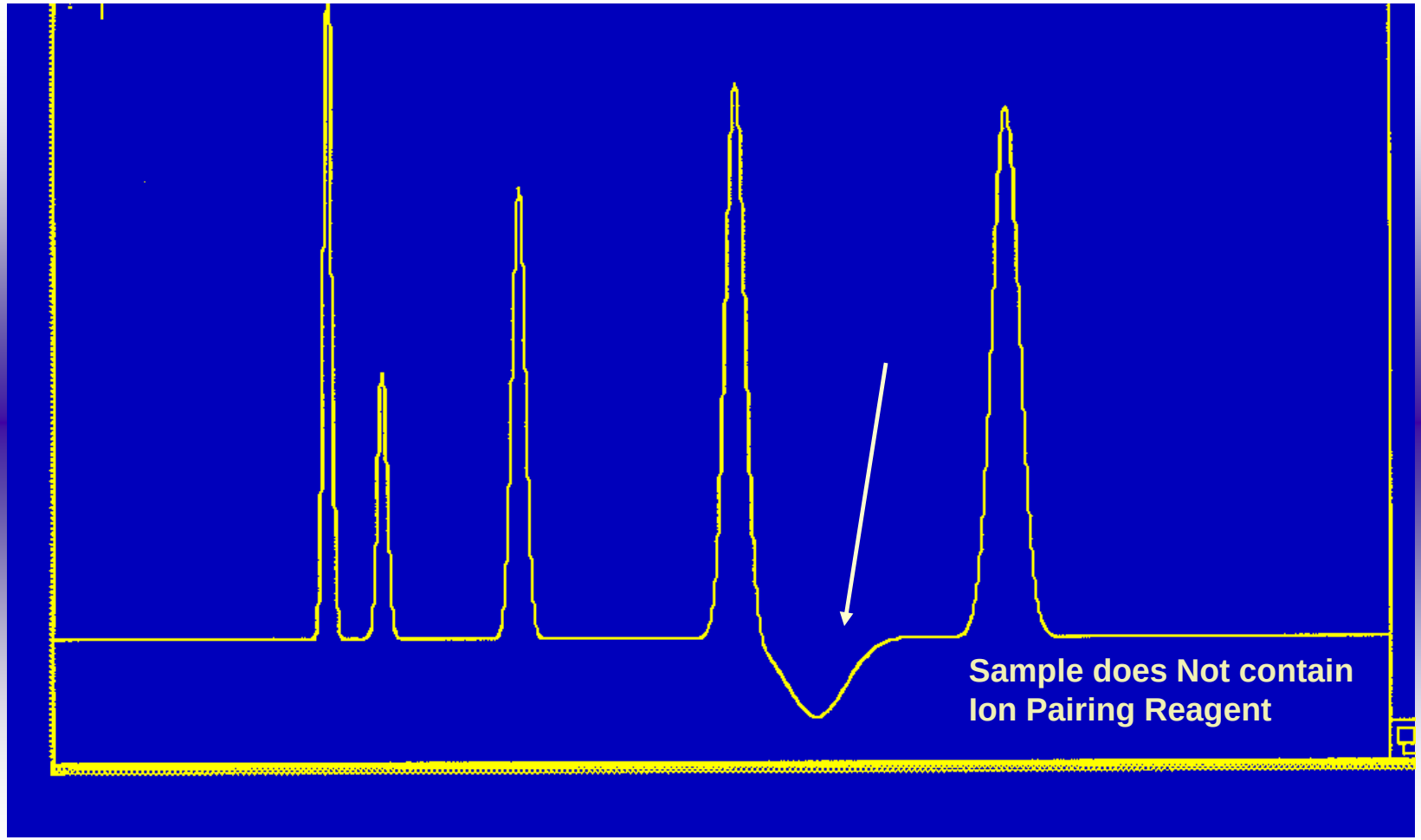
* If mobile phase concentration of PIC reagent is 1 mM/litre, then 600 mL of solvent is needed

*** 600 minutes @ 1mL/min ***
(10 hours)

Waters

Isocratic LC - Negative Peak

Common for Ion-Pairing -- *Sample Solvent*



Troubleshooting Strategy

Problem

Try to simplify --
assess impact on lab efficiency --
inspect the chromatography --
try to categorize --
troubleshoot the easiest to fix items first

CHEMISTRY

- **COLUMN**
  - **GUARD COLUMN**
- **MOBILE PHASE**
- **SAMPLE/VIALS**

MECHANICAL

- **PUMP**
- **INJECTOR**
- **DETECTOR**
- **DATA COLLECTION**
- **BAND SPREADING/ CONNECTIONS**
- **COLUMNS**
- **SAMPLE VIALS**

Waters

Retention Time Problems

- **Reproducibility** &

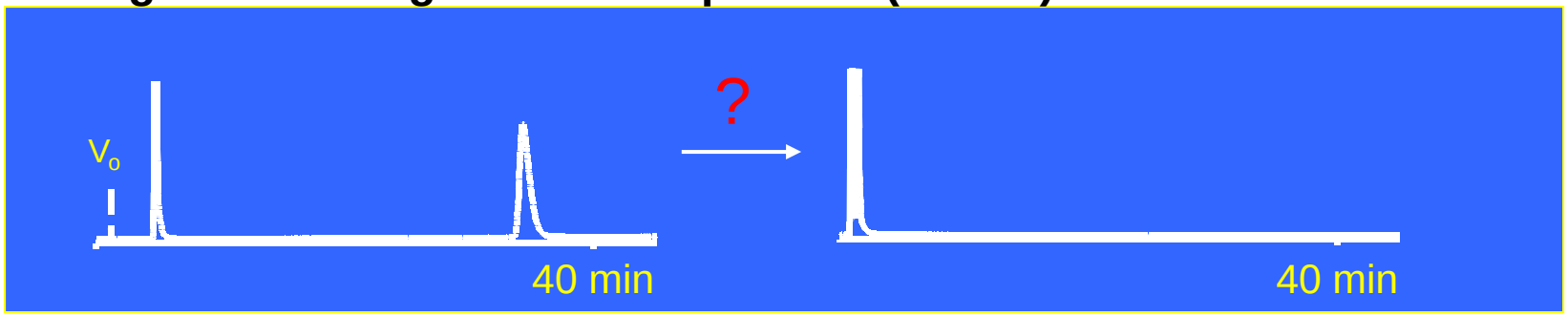
  - Solvent Composition
  - Temperature
  - pH-Control
  - Ion Pairing
  - **Methods Development**

Drifting Retention

- Equilibration
- Stationary Phase Stability
- Column Contamination
- Hydrophobic Collapse
  Low organic < 5%

Hydrophobic Collapse
(Low % Organic solvent)

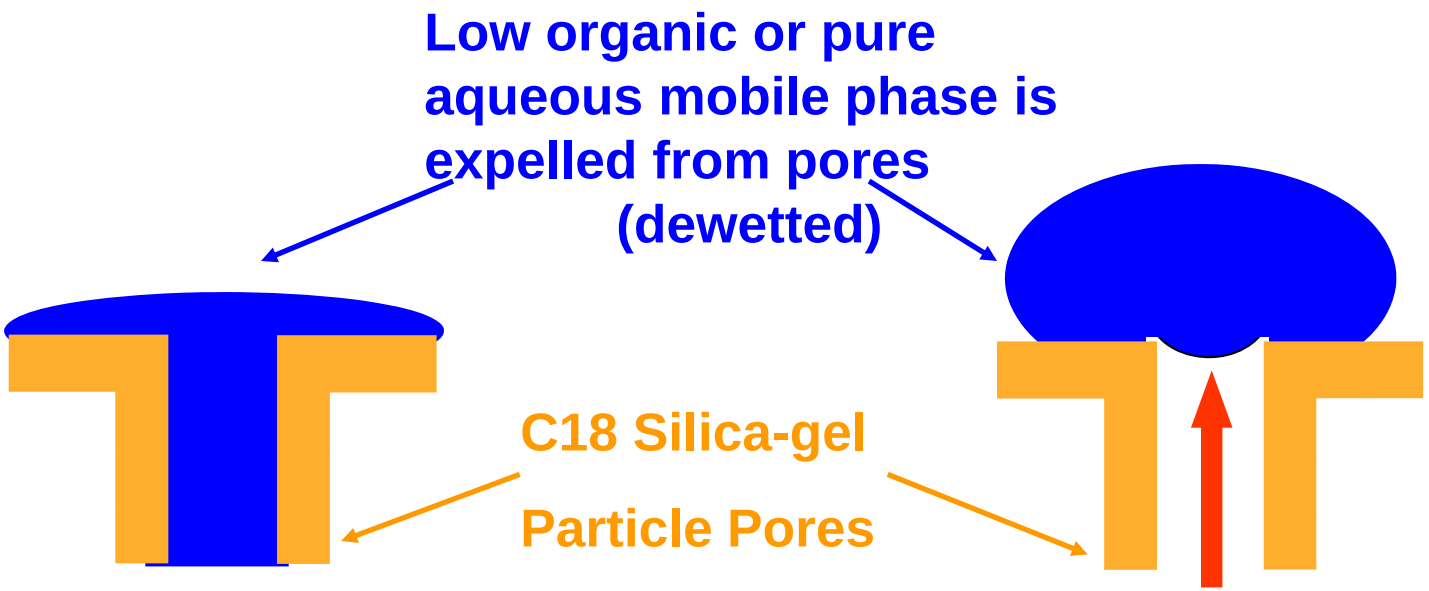
- **Chromatographers have observed complete loss of retention when working with low organic mobile phases. (Polars)**



- **When this phenomenon was investigated it was found that retention times were stable for over 20 hrs (77 injections) using 100% aqueous mobile phase.**

- **However, when the flow was stopped, then restarted, retention was lost. This observation suggested that the mobile phase was extruded from the pores when pressure was released from the column**

What is “Hydrophobic Collapse?”

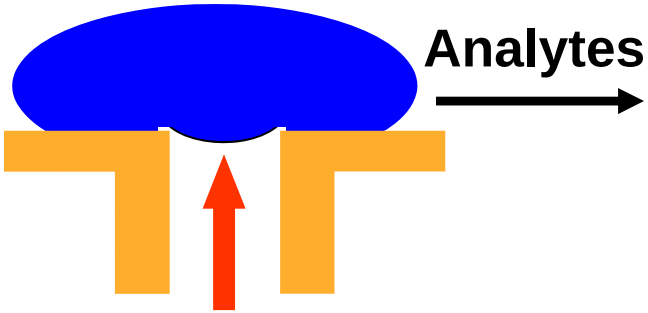


Note: Retentivity is a function of the surface area and ligand density. However, if the surface is non-wetted, then the effective chromatographic surface area is reduced > 95%. Therefore, reducing the retentivity of the analyte --> poor capture = Hydrophobic collapse

Remember all most all of the surface area is in the pores!

How does flow stoppage cause this problem in an HPLC Column? -- Possible Mechanism

Flow stoppage may relieve the pressure that was forcing the aqueous mobile phase into the pores. When the pressure goes down, the hydrophobic pore surface can expel the polar mobile phase and "dewet" the pore.

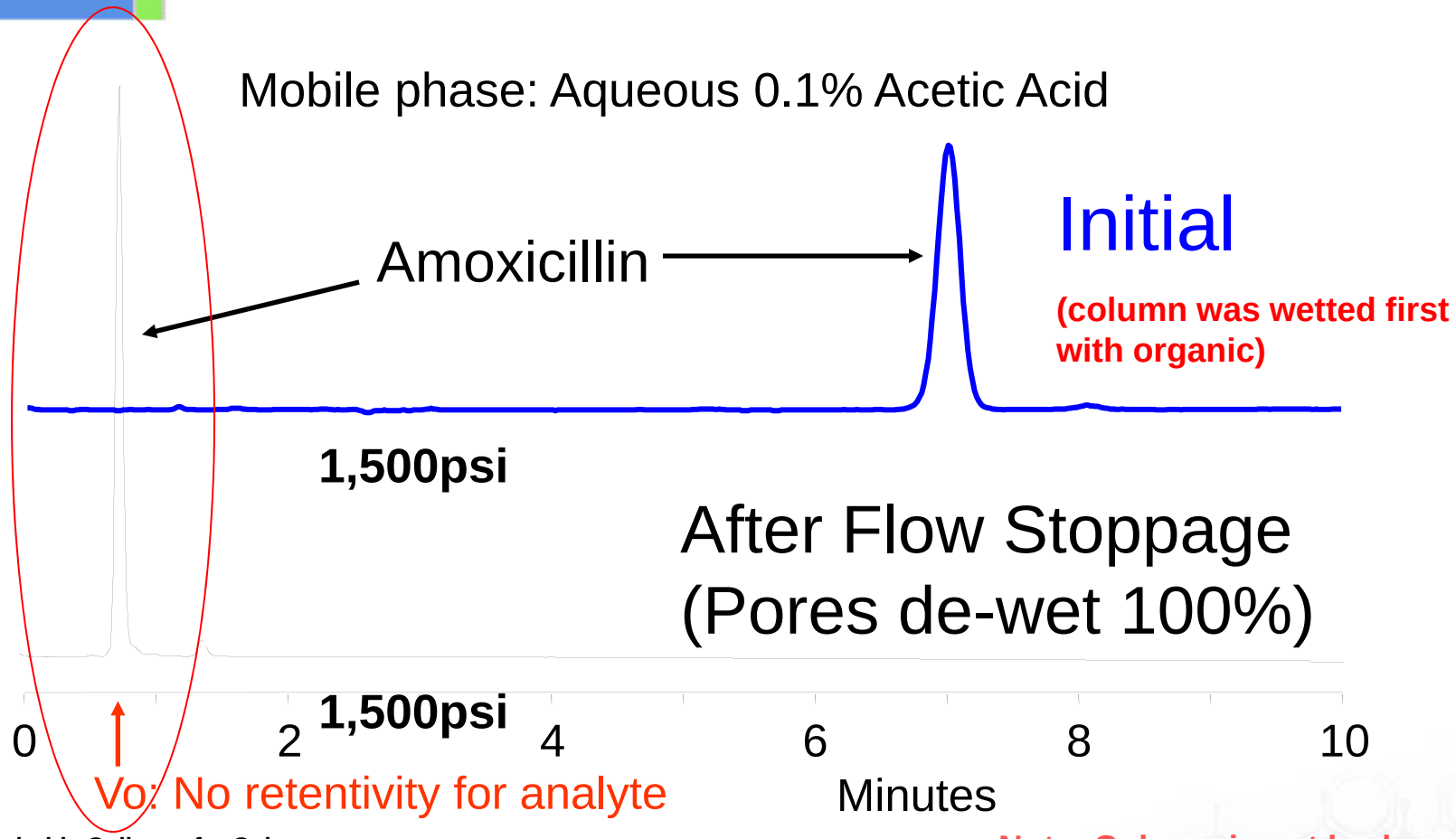


At flow with pressure on the mobile phase.

Stopped flow with no pressure on the mobile phase -- pores de-wet -- restart flow -- pores still de-wettted analytes never enter pores - resulting in no retention. (Need ~ 40% MeOH to re-wet pores)

Note: Packings with Embedded Polar Group Ligands do not show Collapse

High Carbon C8 Column "Hydrophobic Collapse -- HPLC"



Note:Hydrophobic Collapse for Columns Discussed in CCD Column Seminar Series Part 2

Note: Column is not broken -- just stopped working -- Rewet >> ok

Waters

Re-wetting a Stationary Phase Once De-wetted:

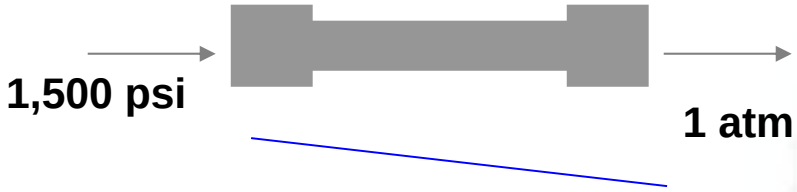
- Use a mobile phase containing > 40 % methanol or other polar organic solvent

  (other organic solvents may vary in % required for wetting)

  - This works by reducing the contact angle

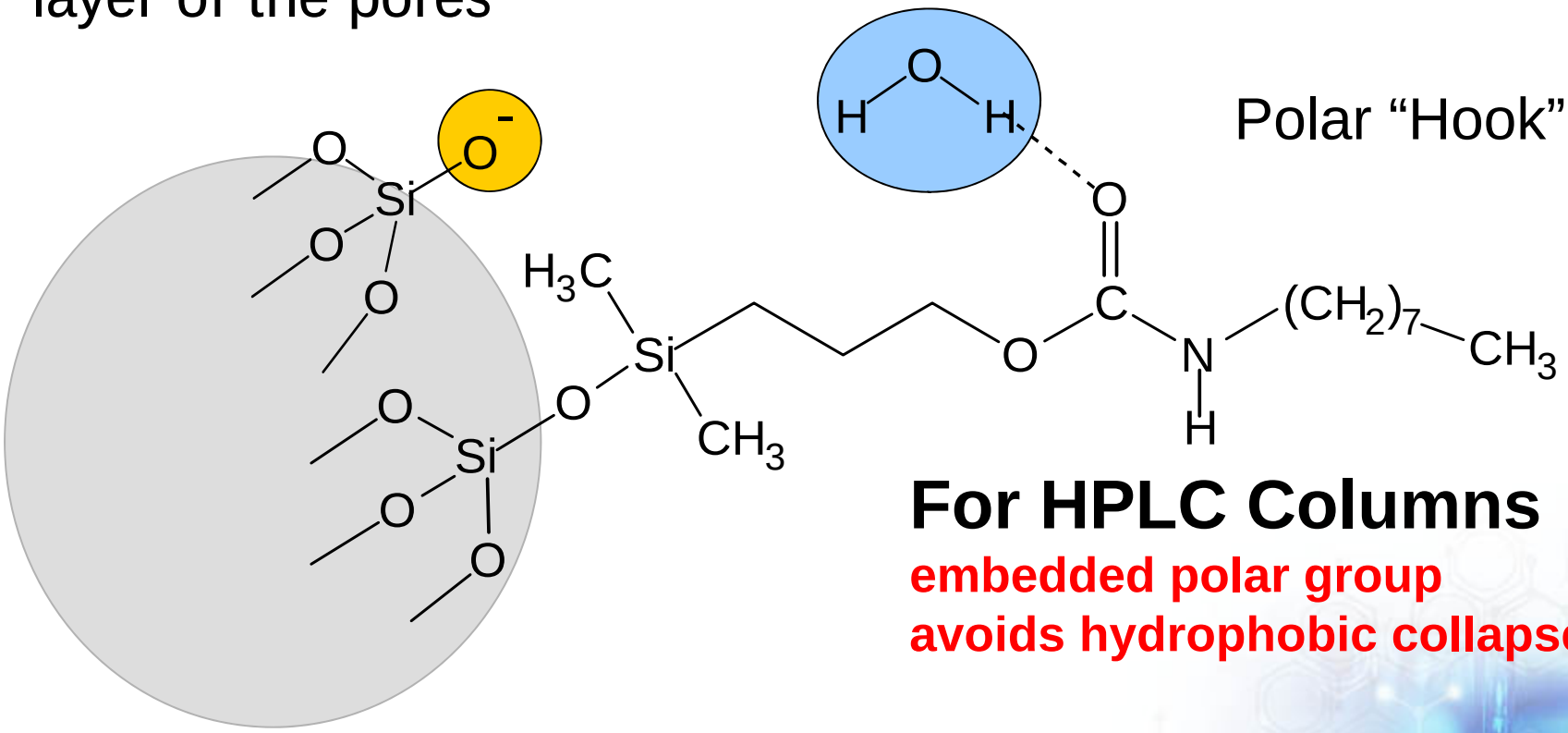
- Do not use pressure to force aqueous mobile phase back into pores

  - Not practical because column outlet is at atmospheric pressure



Embedded Polar Ligand: Reduces Hydrophobic Collapse

Polar group increases water concentration on the surface layer of the pores



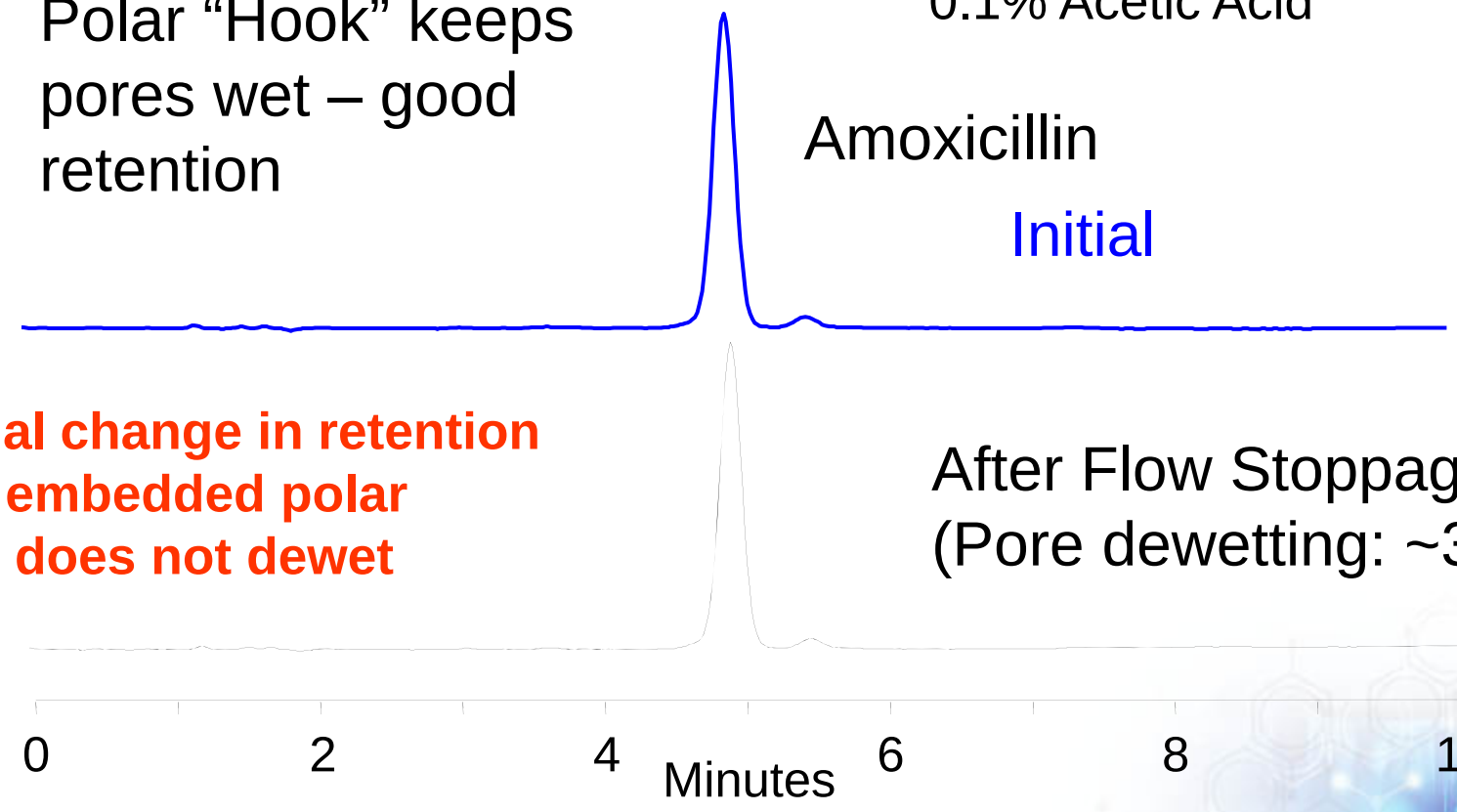
SymmetryShield™ RP8: No Hydrophobic Collapse

Mobile phase: Aqueous
0.1% Acetic Acid

Polar “Hook” keeps pores wet – good retention

Amoxicillin

Initial

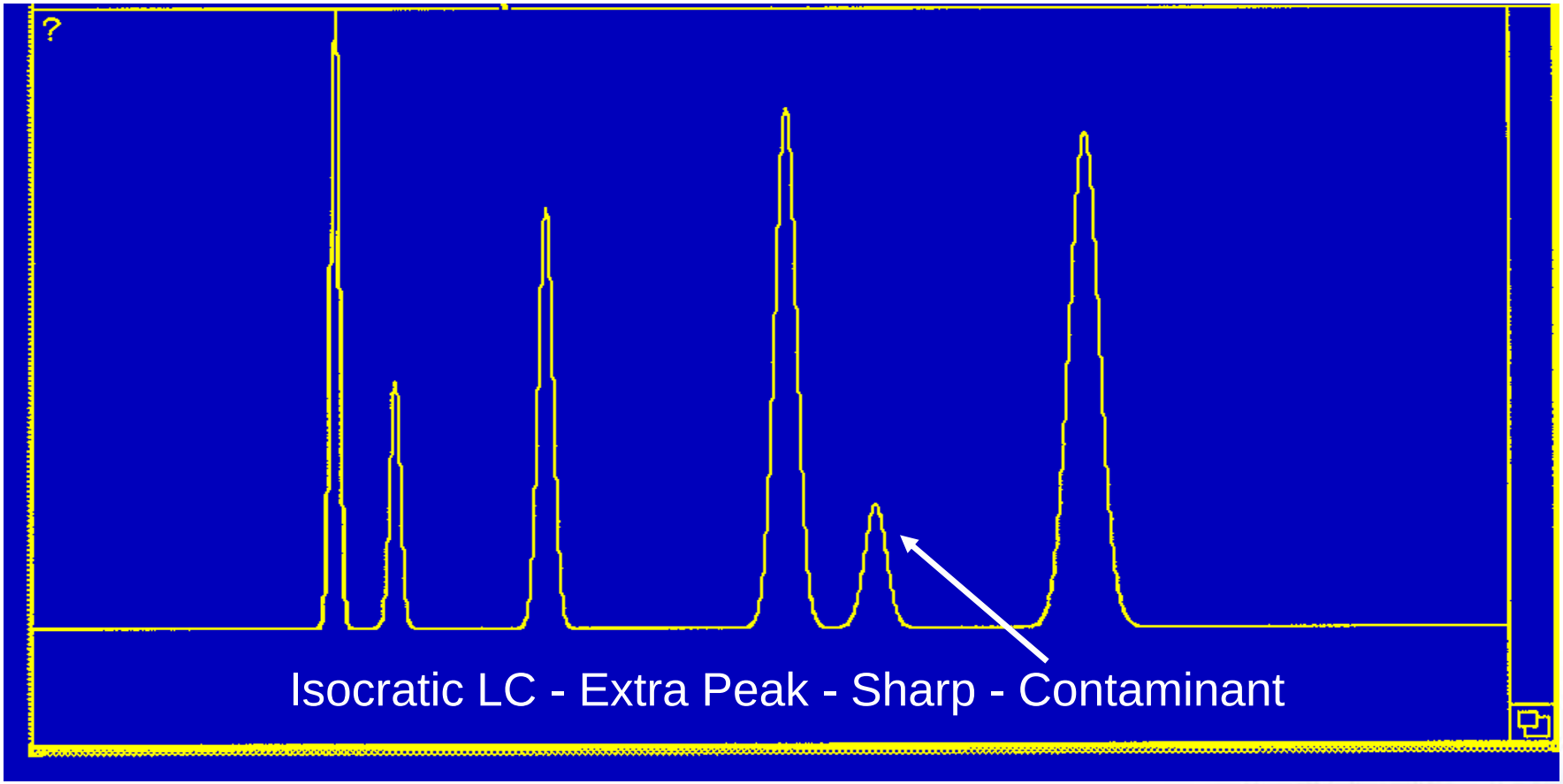


Minimal change in retention time - embedded polar group does not dewet

After Flow Stoppage
(Pore dewetting: ~3%)

Waters

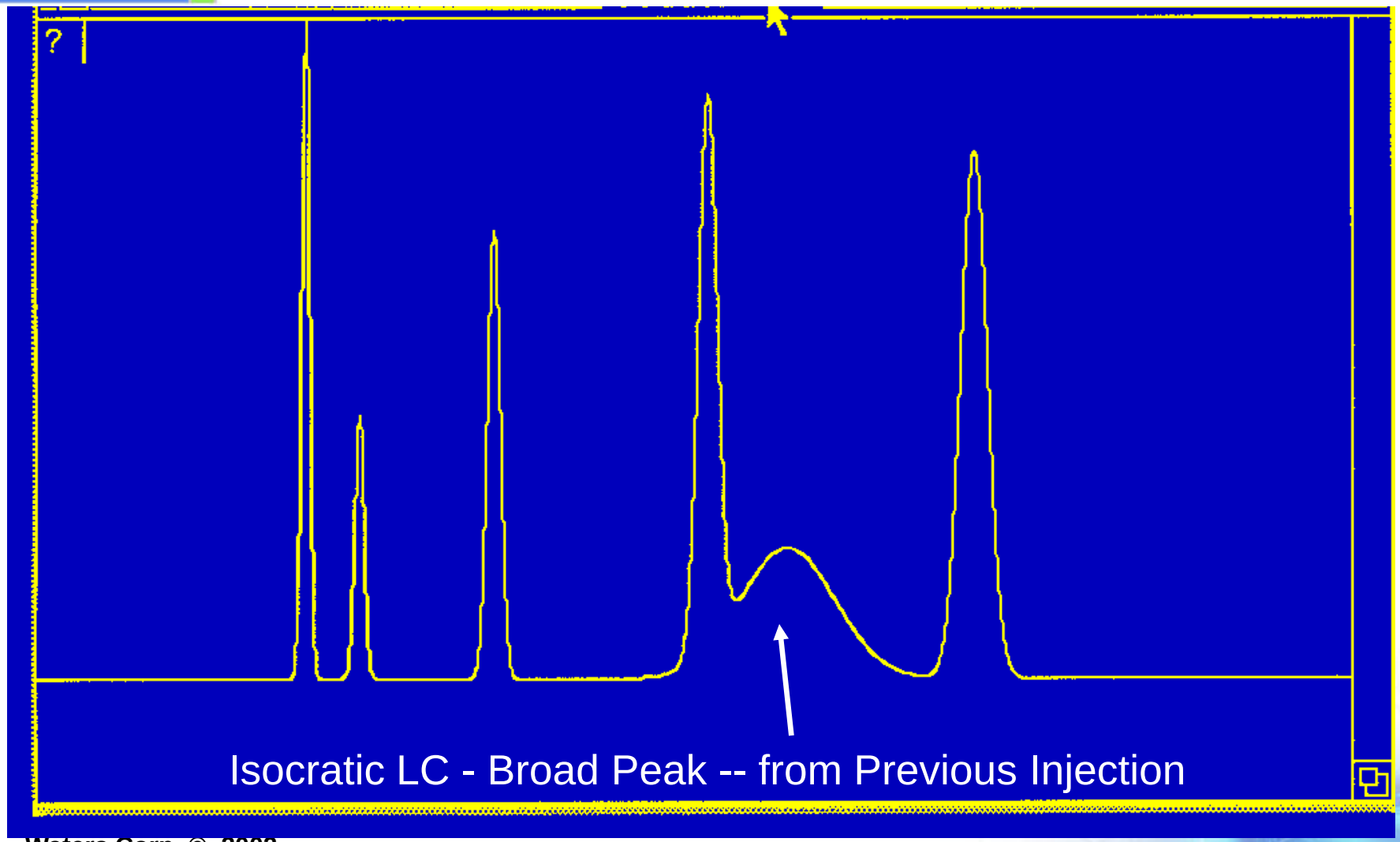
Extraneous Peaks



Could be from; sample, vial, septum etc.

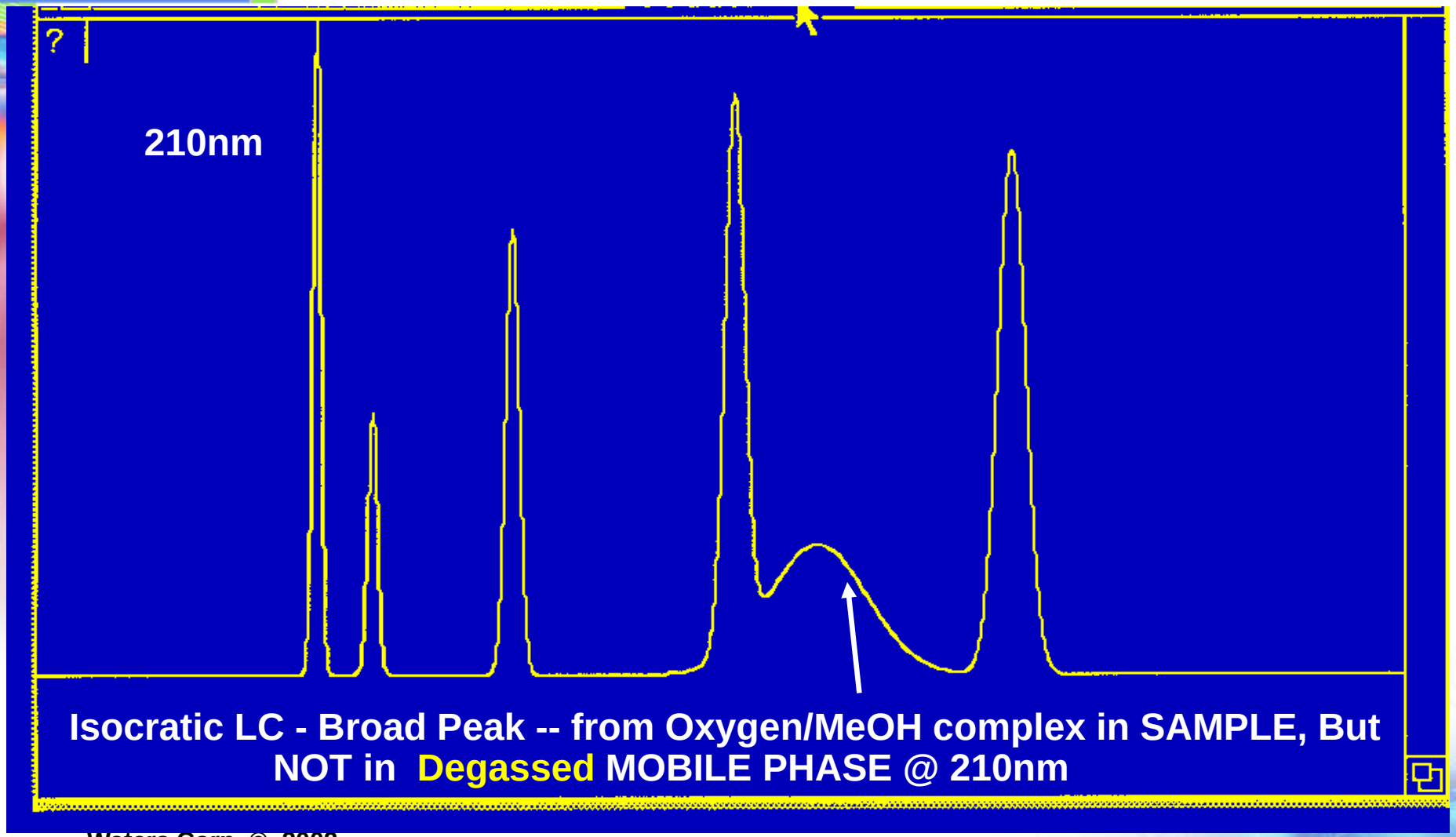
Waters

Extraneous Peaks



Waters

Extraneous Peaks



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Synchronous Noise

SYNCHRONOUS NOISE

ALMOST ALWAYS CAUSED BY THE PUMP

Air in pump head - Prime pump and degas solvent

Check valve problem - Rebuild or replace

Broken plunger - Replace (blame it on someone else)

Mixing problem - Increase system volume

Electrical noise - Change circuits, remove source

Waters

Asynchronous Noise

ASYNCHRONOUS NOISE

BUBBLES

Degas mobile phase

GAS CAUGHT IN DETECTOR

Degas mobile phase. Put backpressure on cell.

LEAKS

Fix leaks, replace fittings

MIXING PROBLEMS

Increase system volume

PLUGGED LINES

Remove plug, flush system

ELECTRICAL PROBLEMS

Remove source, change circuits

Mobile Phase Degassing

Best Solution:

Consider an in-line degassing accessory unit for your HPLC system. Some modern HPLC systems contain a built-in degasser (e.g., Waters Alliance™ System).

Alternatives:

- **Helium sparging – be careful not to alter mobile phase composition by evaporation of volatile components.**

- **Offline vacuum degassing in an ultrasonic bath – incomplete method which provides short-lived degassing.**

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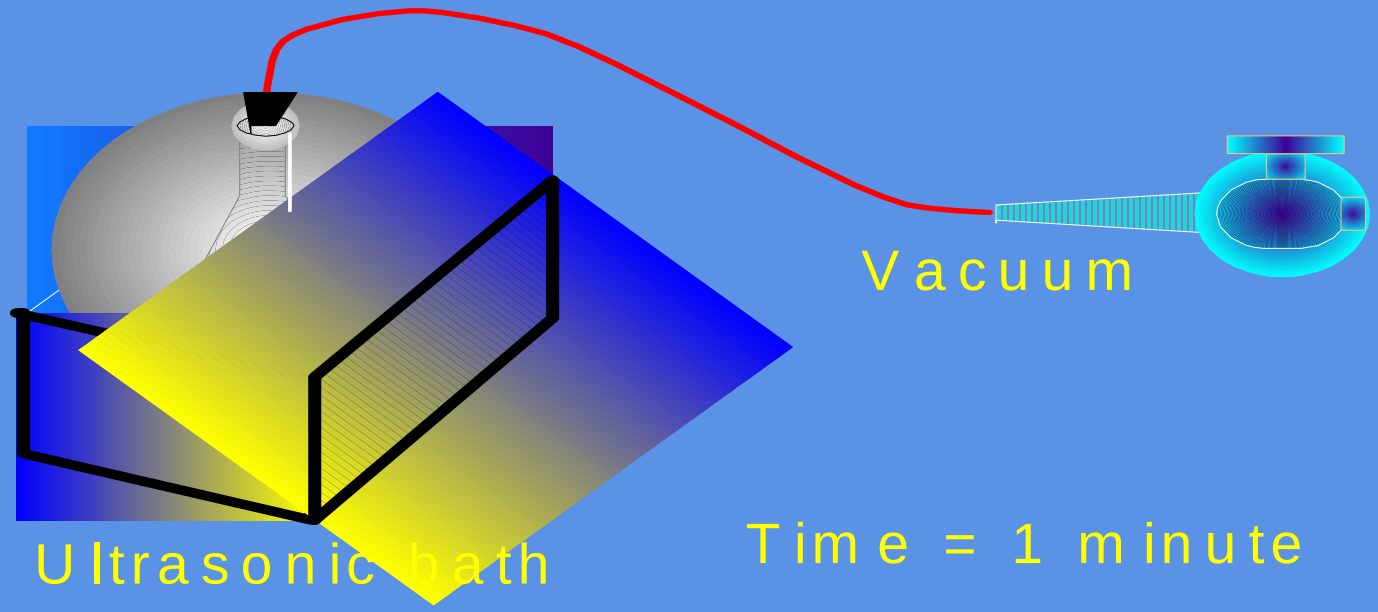
Degas Solvents



Time = 1 minute

Solvent Degassing Precautions

1. Degas solvents prior to adding modifiers
2. Helium sparge is good, as long as solvent doesn't change due to volatility of solvents and/or additives
3. Solvents should be degassed daily

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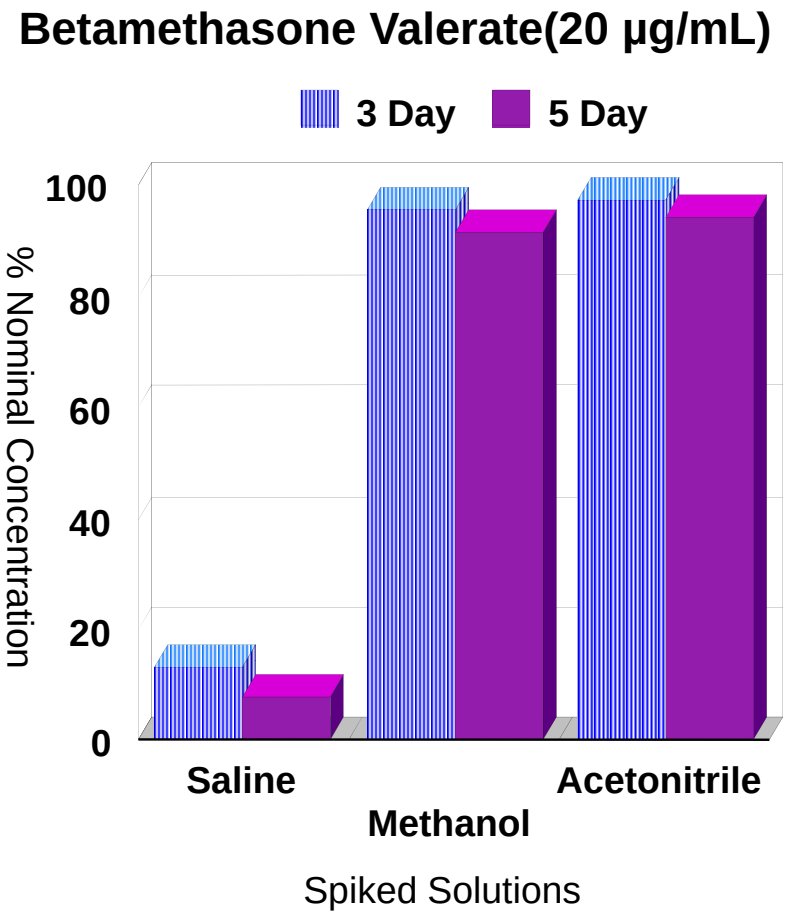
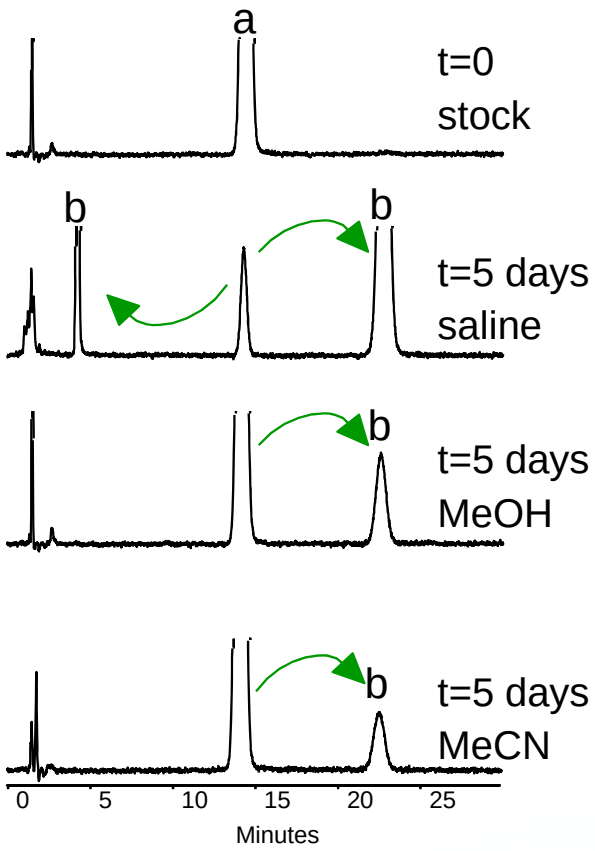
Samples

- **Sample Solubility**
  - **Test in MOBILE PHASE**
    (Avoid precipitation – plug column)
- **Sample Analyte Stability**
  - **pH**
  - **Light (use amber vials)**
  - **Temperature**
  - **Time/Solvent**

*** Problems associated with Vials**

Room Temperature Stability of Sample in Solutions





a: betamethasone valerate
b: breakdown products **(hydrolysis)**

Test in vials you will be using -- may make a difference

Sample Vials

Vials -- often the last thing considered, but may be the best place to start your troubleshooting

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Choosing the Right **SEPTUM**

- **Wrong choice of Septum may result in :**
  - **Evaporative loss of sample**
  - **Lack of reproducibility for repetitive injections**
  - **Septum coring**
  - **Needle damage**
  - **Septum dislodging**

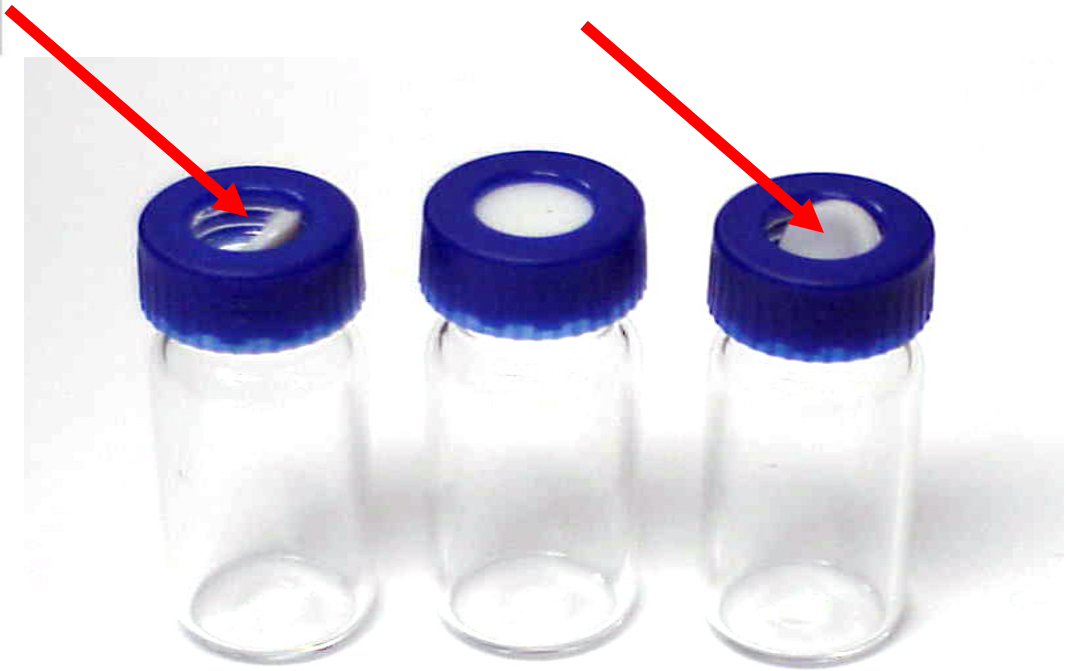
Waters

Choosing the right SEPTUM

Material	Compatibility	Recommendations	Self Sealing	Max. Temp.
PTFE (Teflon)	All solvents; Ideal for MS applications	Only suitable for one injection	No	260°C
PTFE/Silicone	PTFE resistance until punctured. After 1st injection: alcohols, acetone, ether, DMF, DMSO	Recommended for multiple injections and sample storage.	Very good	200°C
PTFE/Silicone pre slit	same as above	Very good for multiple injections. Eliminates vacuum formation and delivers excellent reproducibility. Prevents coring from bottom draw-port needles.	Good	200°C

Note: 1) Use Self Sealing Septum to minimize evaporative loss of volatiles
2) PTFE/Silicone septum – always have the PTFE side to the sample to reduce extractables

Problem - Septum Dislodging



Common problem with Non-bonded septum -- Can jam autosampler

- **Don’t immediately blame the needle or needle alignment**
- **Switch to preslit septum**
  - **Less force required to pierce**
- **Choose bonded septum (LectraBond™)**
  - **Septum is bonded to the cap. Eliminates dislodging**
    - **Electron welded; no chemicals or adhesives**

Problem – Sample Draw Volume Reproducibility -- Venting

- **Symptom: Peak area increases after first injection from the same vial (first injection – low, latter injections OK)**
  - **Possible cause: Inadequate venting upon needle piercing the septum/cap for the FIRST Time. (Vacuum formation) Caused by the septum/cap sealing around the injection needle -- vacuum draws some sample back out of the needle**
    - **Situation can be aggravated by over filling the vial (Never fill the vial all the way to the top)**
    - **Test: remove cap and septum from vial, perform multiple injections, measure peak area to determine if the septum/cap is the cause. ***

***Some auto samplers may have to have the vial sensor defeated**

Problem – Sample Draw Volume Reproducibility -- Venting

- **Solutions:**
  - **If using self sealing PTFE/silicone septum:**
    - **Switch to preslit PTFE/silicone septum**
      - **Preslit septum will provide adequate venting with good resealing capability**
    - **Switch to PTFE septum**
      - **PTFE will tear upon piercing and provide adequate venting. However it will not reseal**
  - **If using solid closure (star burst design)**
    - **Available from a variety of suppliers and vary dramatically in performance.**
      - **contact the HPLC instrument supplier to find a cap designed for your HPLC system**

Problem – Sample Draw Volume Reproducibility – Coring of Septum

- **Symptom: Peak area varies (increases /decreases) from injection to injection from the same vial**
  - **Possible cause:Coring of Septum by Needle If using a bottom draw port needle, the draw port could be plugged with septum material**
    - **Check needle draw port for septum material, remove / replace.**

Solution: if using self sealing PTFE/silicone septum:

- **Switch to preslit PTFE/silicone septum**
  - **Preslit septum will eliminate coring and deliver good resealing capability**
- **Or, Switch to PTFE septum**
  - **PTFE will eliminate coring. However, it will not reseal**

Silicone Septum Material Lodges in Bottom Draw Needle

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Problem -- Spurious Peaks

- **Contaminants can come from three sources and can be either sample dependent or detection-method dependent**
  - **Vial**
  - **Cap**
  - **Septum**
    - **Most common contributor**
      - **Chemical compatibility**
      - **Absorbs contaminants from atmosphere**
      - **Observance of problem can be detection-method dependent**

Waters

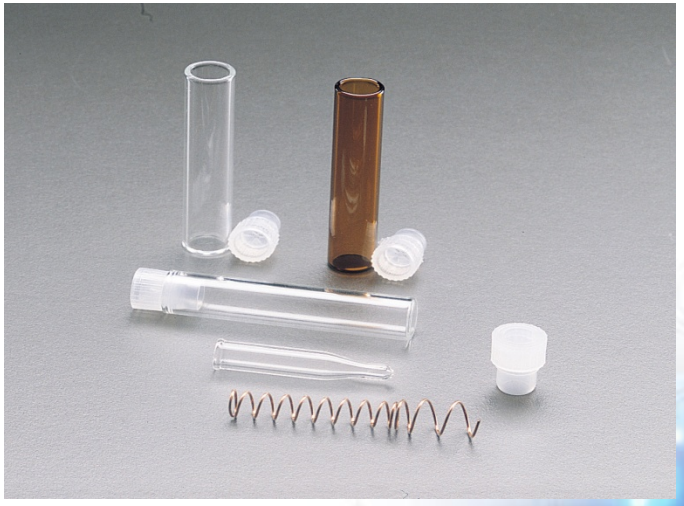
Problem -- Spurious Peaks

- **Simple, quick test**
  - **Remove cap and septum from vial, run sample to see if spurious peaks still appear.**
    - **If peaks don't appear, the problem is coming form the septum**
      - **Switch to a PTFE septum (most chemically inert material)**
- **If peak still appears, troubleshoot the vial**
  - **Vials are manufactured from different grades of glass. Choose a vial manufactured from a different grade / class of glass**
  - **Packaging used for shipping the vials can also contribute to this problem. Choose a vial from an alternative supplier**

Choosing the Right VIAL –
Excessive residual sample volume

- **When sample volume is limited and / or injection volumes are very low, traditionally, analysts use Low Volume Inserts (LVIs)**

Additional pieces to buy and to handle
Sample spillage
Proper fit of LVI
Limited capacity
Narrow neck opening / difficult to fill



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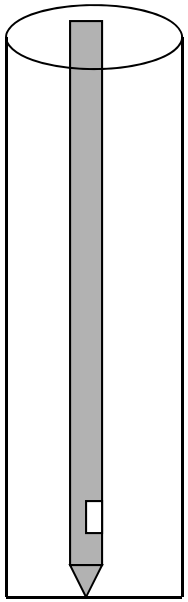
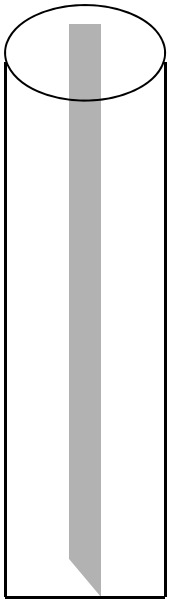
Problem -- Excessive Residual Sample Volumes

- **Problem: inability to draw all the sample from the vial**
  - **Function of the needle design and the internal vial shape**

Side Draw

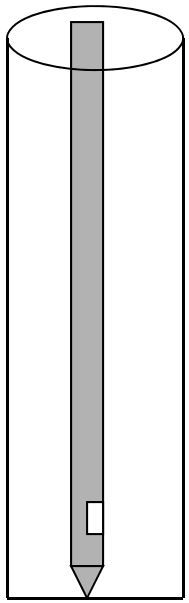


Bottom Draw



Problem -- Excessive Residual Sample Volumes

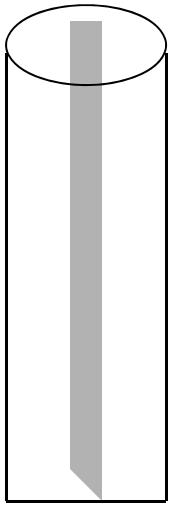
- Needle Design



- Side Draw Port
  - Draw port in located along the needle shaft, not at the base of the needle
    - Benefit: Eliminates coring
    - Drawback: Unable to draw sample below the draw port location
  - Use vial with internal taper designed for side draw port needles
    - Waters Total Recovery™ vials
    - QsertVial™
    - Low Volume Inserts

Problem -- Excessive Residual Sample Volumes

- **Needle Design**
  - **Bottom Draw port needles**



- **Benefit: Higher level of sample draw**
  - **Still will not draw all the sample out of a flat bottom vial**
- **Problems: Coring and sample draw volume reproducibility caused by bottoming out**
- **Use vial with internal taper designed for bottom draw port needles/preslit septum**
  - **Waters Maximum Recovery™ vial**
  - **QsertVial™**
  - **Low volume insert**
  - Use vials recommended by the instrument manufacturer

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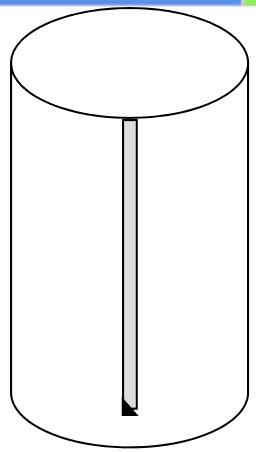
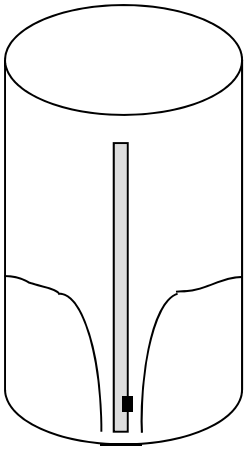
Problem -- Excessive Residual Sample Volumes



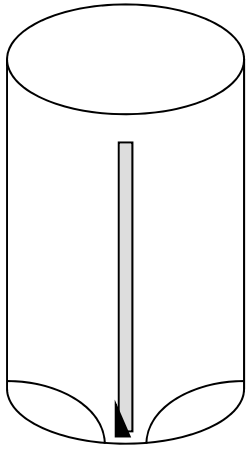
Bottom Draw

Standard



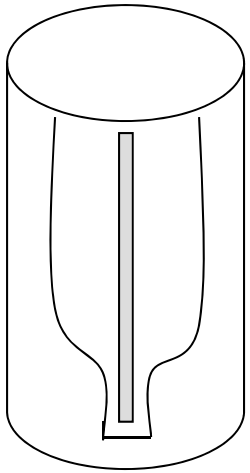
Side Draw

Total Recovery



Bottom Draw

Max Recovery



QSert

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APPENDIX

- Column Installation and Equilibration
- Column Protection
  - Guard Columns
  - In–line Filters
  - Solvent Viscosities
- Column Storage
- Band Spread Test / Plate Count
- Buffers

Column Maintenance Information

- **Care & Use Manuals**

  - Good Source for Information
  - Guidelines (Care, Use, Storage)
  - Help Identify Problem

- ***"Not Necessarily Column Related"***

  - Troubleshooting Tips

Installation and Equilibration

- Purge column with 10 column volumes of mobile phase to be used in analysis (4.6x150mm >=25mL)(see table -- next page)

- Reversed-Phase (C_{18}, C_8 etc.) columns equilibrate faster than Normal Phase columns
  - (order of magnitude = 10)

- Normal phase columns (silica or alumina) may take several DAYS at flow rates of 1.0 ml/min

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Installation and Equilibration

Internal diameter (mm)	Length (mm)	Volume (ml)
2.0	150	.47
2.0	300	.94
3.9	50	.6
3.9	75	.9
3.9	100	1.2
3.9	150	1.8
3.9	300	3.6
4.6	150	2.5
4.6	250	4.2
5	100	2.0
8	100	5.0
7.8	300	4.3
19	150	43
25	100	49
30	300	212
40	100	125
47	300	520
50	300	589

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Column Protection

Waters

Column Maintenance

HPLC columns require relatively little care.

However, they can be damaged if:

1. Dropped on the ground
2. Banged around (drawer)
3. Stored in the freezer/ refrigerator (depends on type of solvent)

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Performance Monitoring

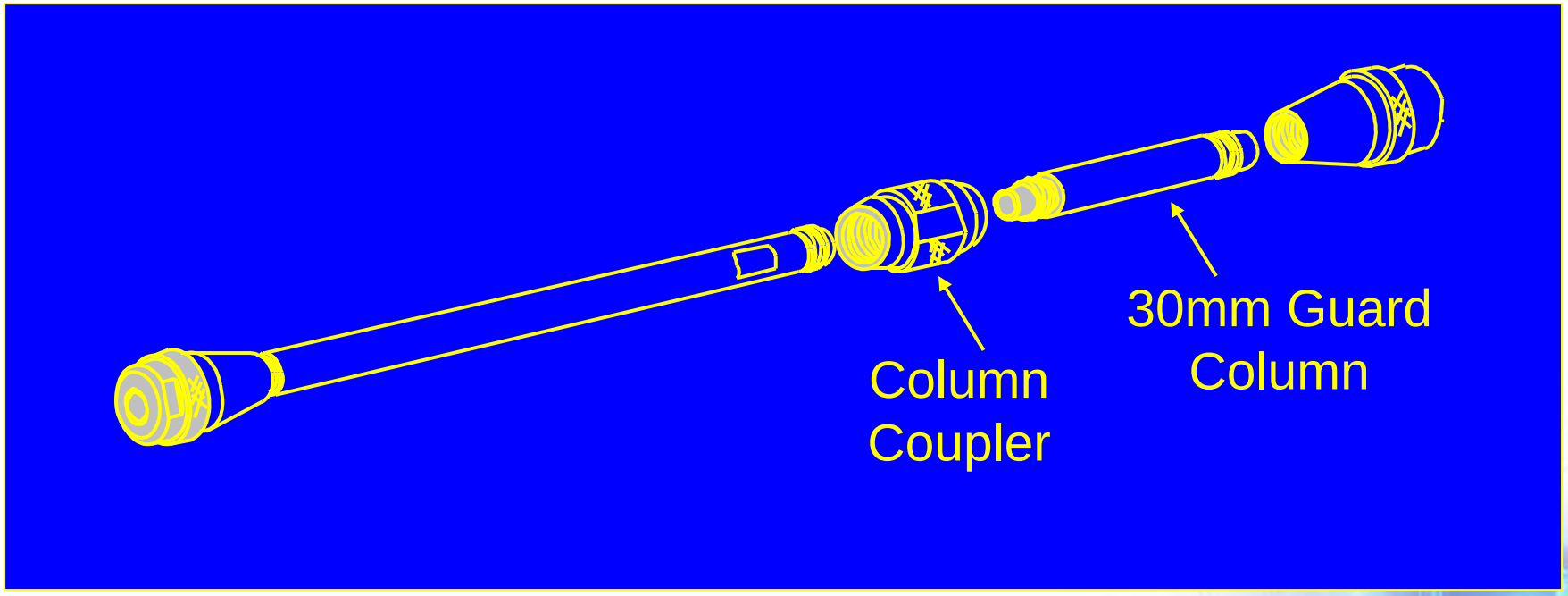
HPLC columns have a finite lifetime.

Deterioration may occur due to:

- **Contamination from adsorbed materials**
- **Debris from instrument seals**
- **Chemical Degradation (hydrolysis) of Column**
- **Particulate Contamination**

Column Protection

Major cause of column deterioration is contamination. Use of guard columns may increase column life-time to > 10,000 analyses



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Column Protection

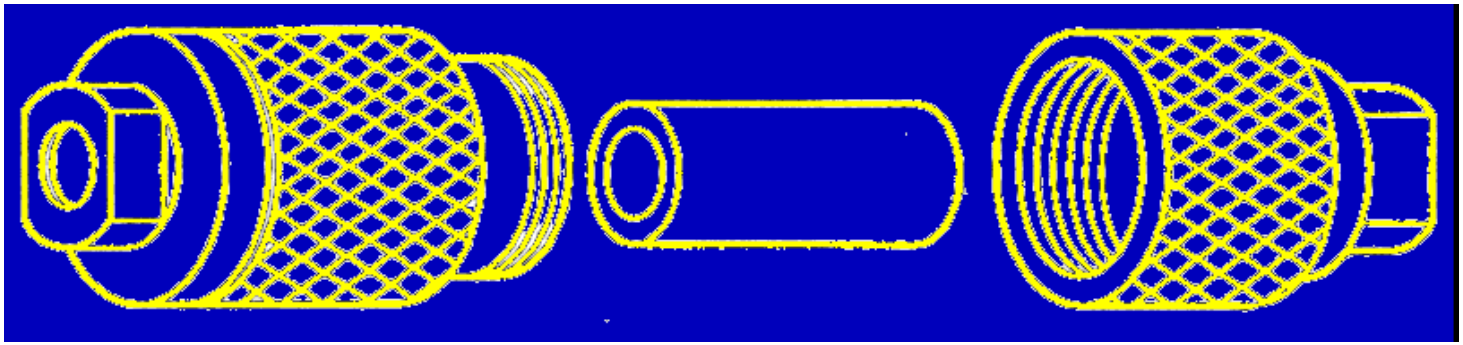
- **Guard column should be regarded as a cost-effective sacrifice to extend analytical column life-time**

- **Should contain IDENTICAL packing material as the analytical column e.g. using a different C_{18}, with different retention properties could actually destroy the separation or impair protection**

Well designed, well packed guard columns will actually IMPROVE the analytical separation efficiency

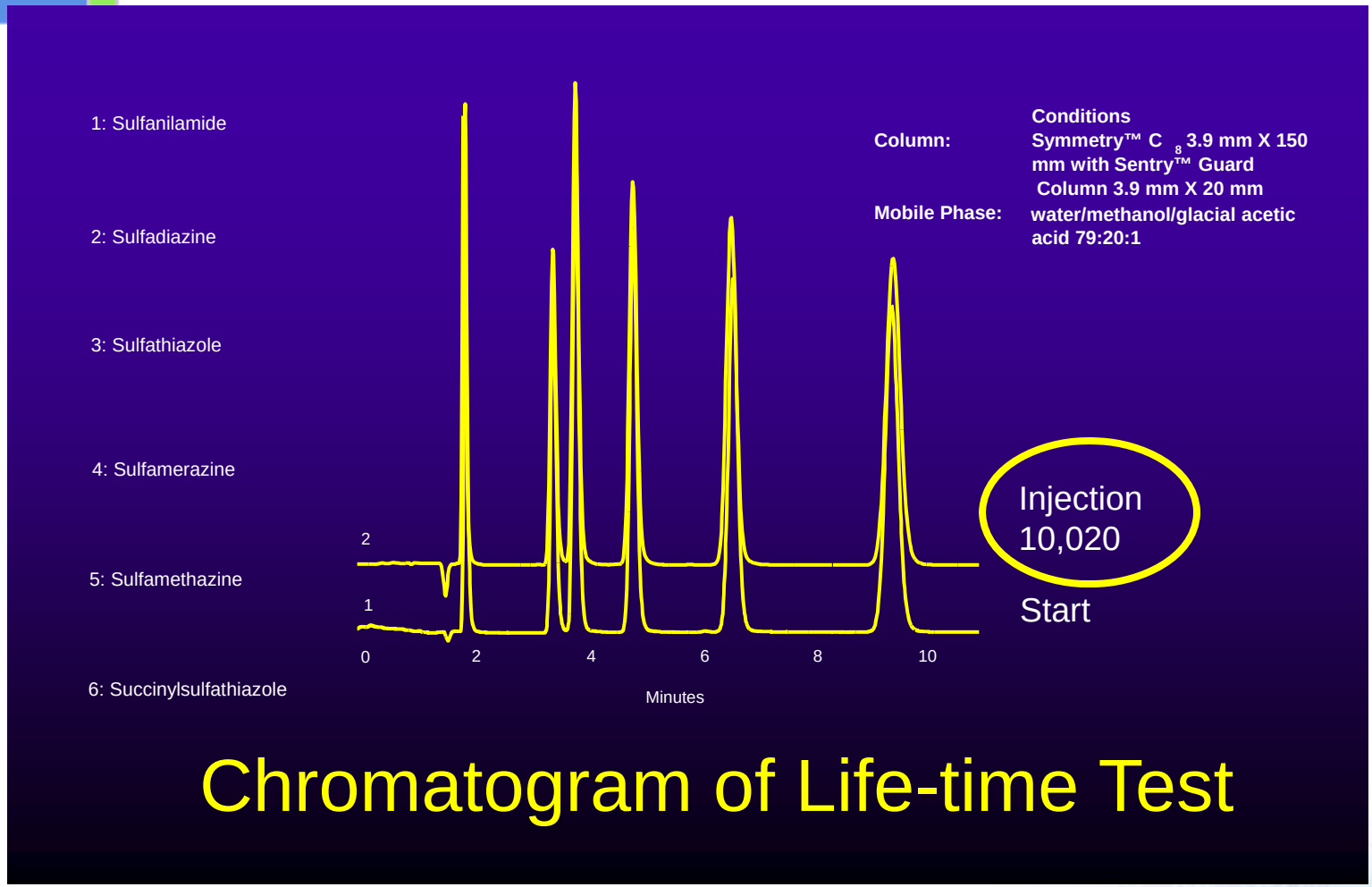
In some cases, SPE may be more desirable

Column Protection



Waters Sentry Guard Column & Universal Holder

Column Protection

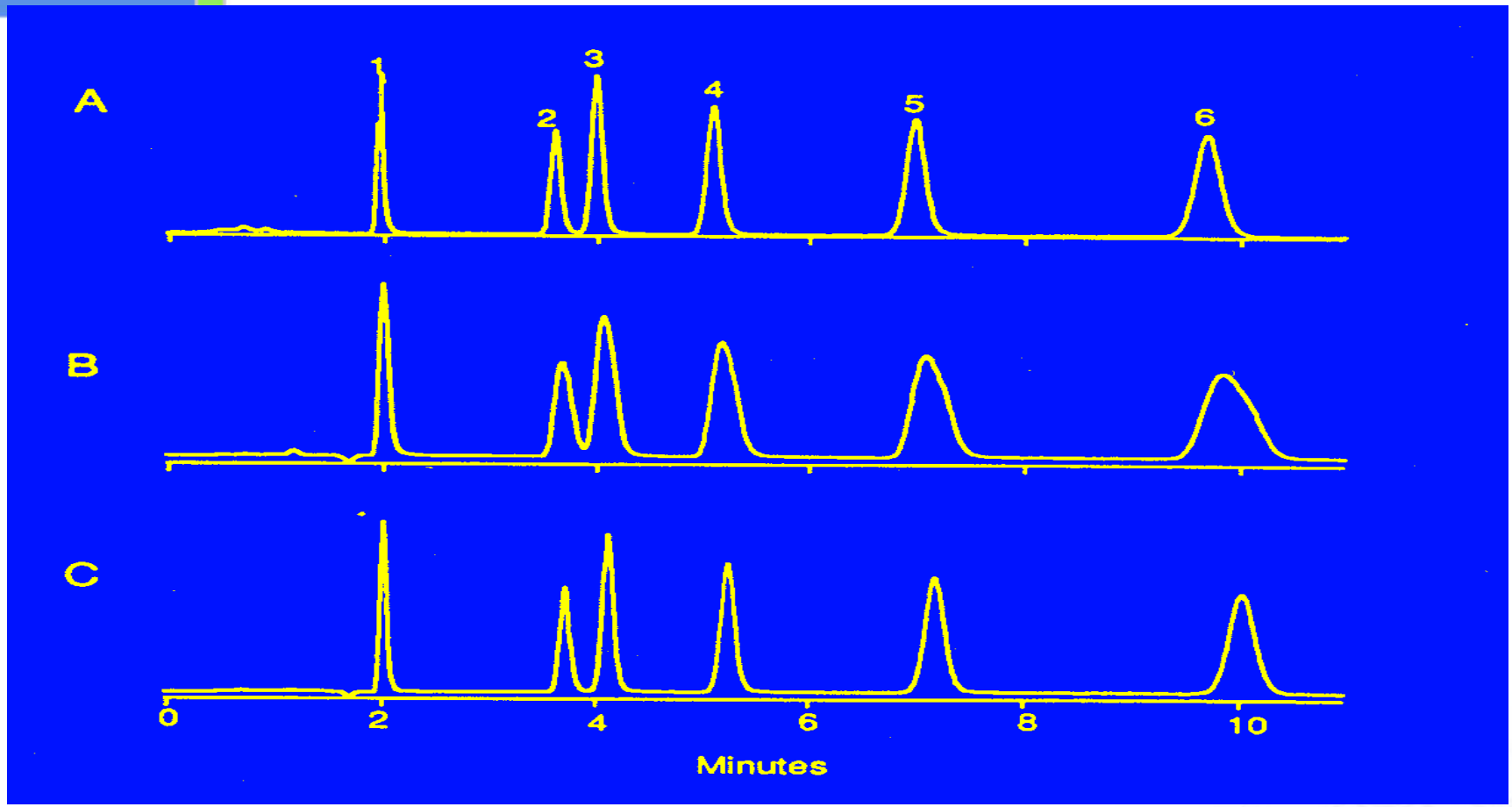


Chromatogram of Life-time Test

Sentry Guard Column Replaced every 500 injections

Waters

Column Protection



Extension of column lifetime with Guard Column using a mixture of sulpha drugs as the sample

A. Initial injection on Symmetry C8 Sentry guard column

B. After 950 injections on same Sentry guard column

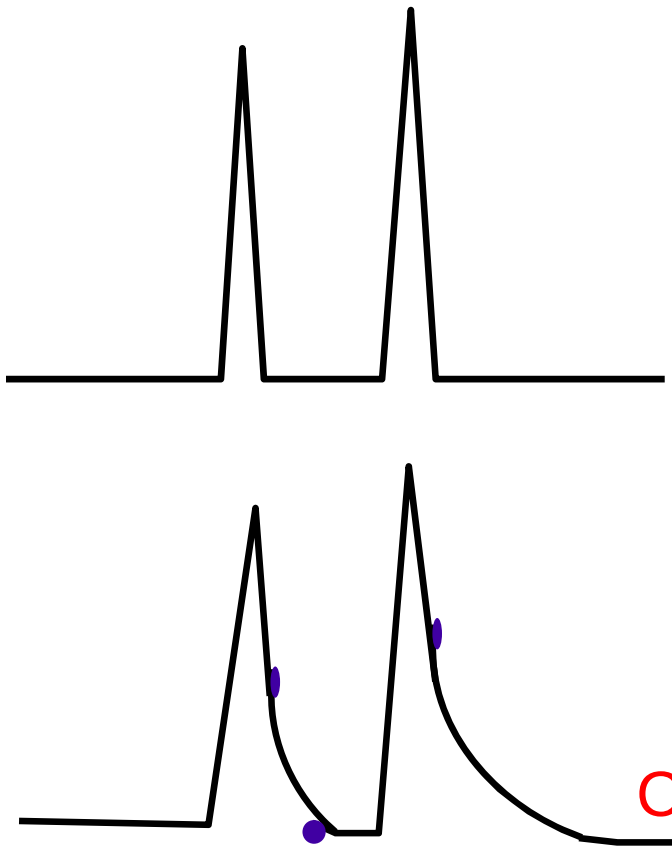
C. New Sentry Guard column for injection 951 on analytical column

Column Protection

Other Techniques to Protect the Column:

- **In-line Filter between the Injector and Column**
- **Filtering of the Sample (Doesn't Protect against Seal Shedding)**
- **Sample Cleanup through Solid Phase Extraction (SPE)**
- **Limit High Back Pressures (solvent viscosity)**

Contaminated In-Line Filter -- Poor Peak Shape



Debris from seal shedding, particulates from buffer, particulates from sample

New Frit = 800 psi

Contaminated Frit = 2500 psi

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Solvent Viscosities

Solvent	Viscosity [cP] at 20° C
Acetone	0.32
Acetonitrile	0.37
Cyclohexanone	0.98
Di-isopropylether	0.37
Diethyl ether	0.23
Dimethyl acetamide	2.1
Dimethyl formamide	0.92
Dimethyl sulfoxide	2.2
Dioxane	1.54
Ethanol	1.2
Ethyl acetate	0.45
Hexafluoroisopropanol	1.0
iso-Propanol	2.5
Isooctane	0.5
Methanol	0.6

Solvent	Viscosity [cP] at 20° C
Methyl acetate	0.37
Methylene chloride	0.44
Methylethyl ketone	0.4
n-Heptane	0.42
n-Hexane	0.33
N-Methyl pyrrolidone	1.67 (25° C)
n-Pentane	0.235
n-Propanol	2.3
o-Dichlorobenzene	1.41
Tetrahydrofuran	0.46
Toluene	0.59
1.2.4-Trichlorobenzene	1.89 (25° C)
Water	1.0
m-Xylene	0.62
o-Xylene	0.81

Waters

Column Regeneration

* Always follow column vendor's guideline for regeneration

* Regeneration can bring back a column's performance if problem relates to compounds, which are retained under method conditions, causing changes in chromatography. Washing them off with more aggressive solvents can return performance

* If surface has been chemically altered, ie hydrolysis of ligands and endcapping, then performance may not be restored

Solvent/Water Mixtures Create High Viscosities -- Back Pressure

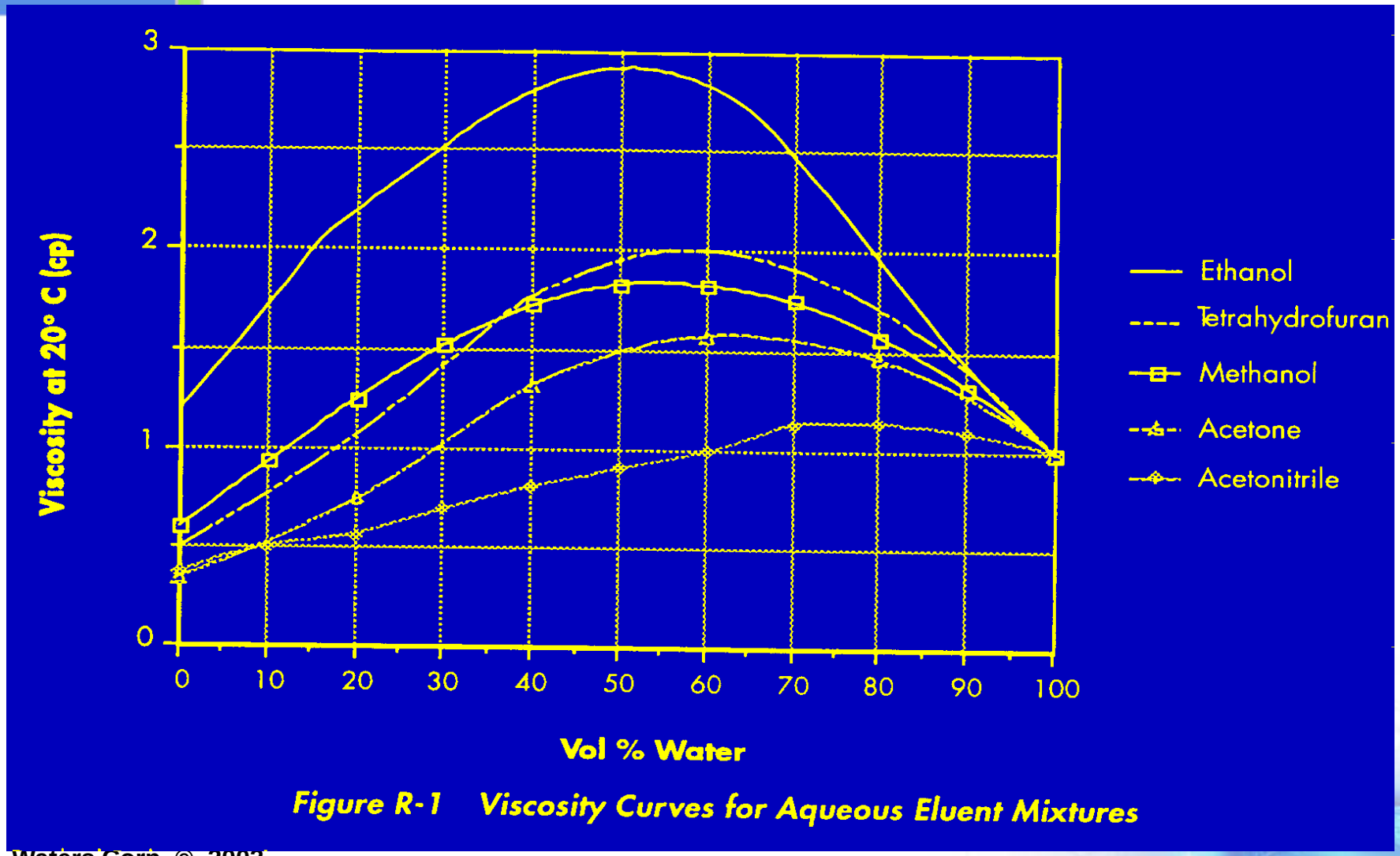

Figure R-1 Viscosity Curves for Aqueous Eluent Mixtures

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Column Use (Silica Based)

- Increasing solubility of silica at high pH
- Chemical instability of bonded phase at low pH
- Elevated temperatures will decrease column lifetime (up to 3 x for 10°C rise)
- C_{18} (ODS) approximately 1000 times more stable than CN (cyanopropyl)

Waters

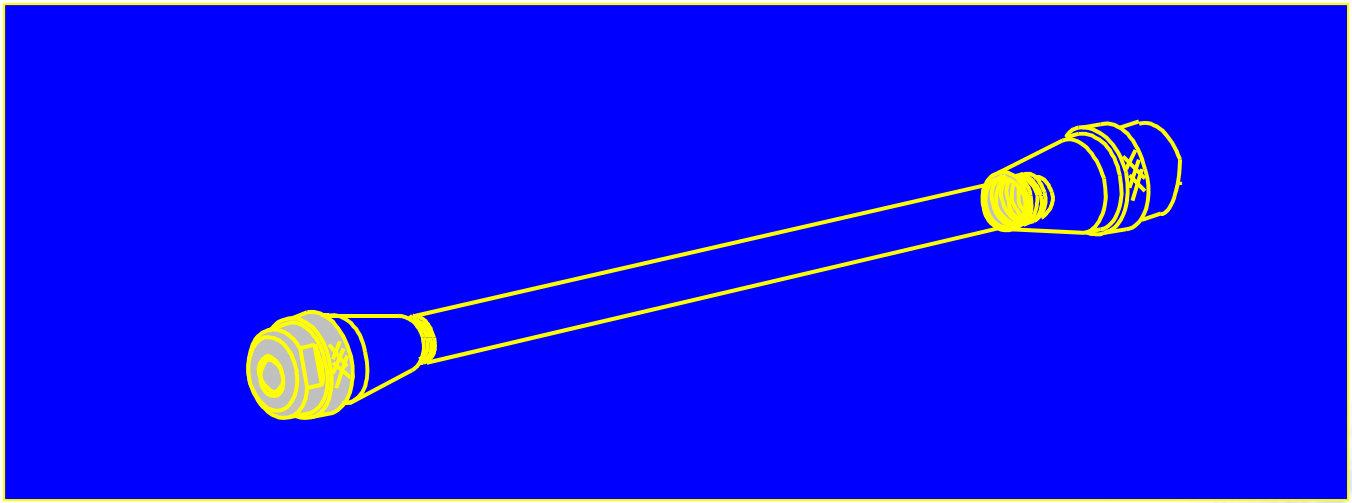
Column Use (Polymer Based)

- Susceptible to pressure 'shock'
  - Require gradual flow increase/decrease
- Compatibility with solvents
  - Expansion vs. Contraction
  - Solvent changeover
  - Aqueous vs. Organic
- Limited range of solvents
  - Packing material dependant

Waters

Column Storage

- **Store in Mobile Phase for Short Periods of Time (<72hrs.)**
- **Store in Shipping Solvent for Longer Periods of Time**



Waters

Column Storage

Column should be stored in solvent which manufacturer recommends

- **For bonded phases, use organic solvent (eg. MeOH or ACN) -- Using non-aqueous solvents minimizes hydrolysis.**
- **Some bonded phases (CN) become unstable in polar organic mobile phases. Storage in water or buffer is then okay.**
- **Worst mobile phase for CN column is CH_3CN**

Column Storage

Columns which may be stored in Water or Buffered Solvents:

- **Ion exchangers**
- **Aqueous SEC packings**

However:

Prevent microbial growth by using 0.05% sodium azide in mobile phase

OR

Small quantity of organic solvent (acetonitrile 5% or methanol 10%)

Waters

Column Storage

* Columns which should be stored in Mobile Phase:

  - Normal Phase
  - Organic SEC (GPC)