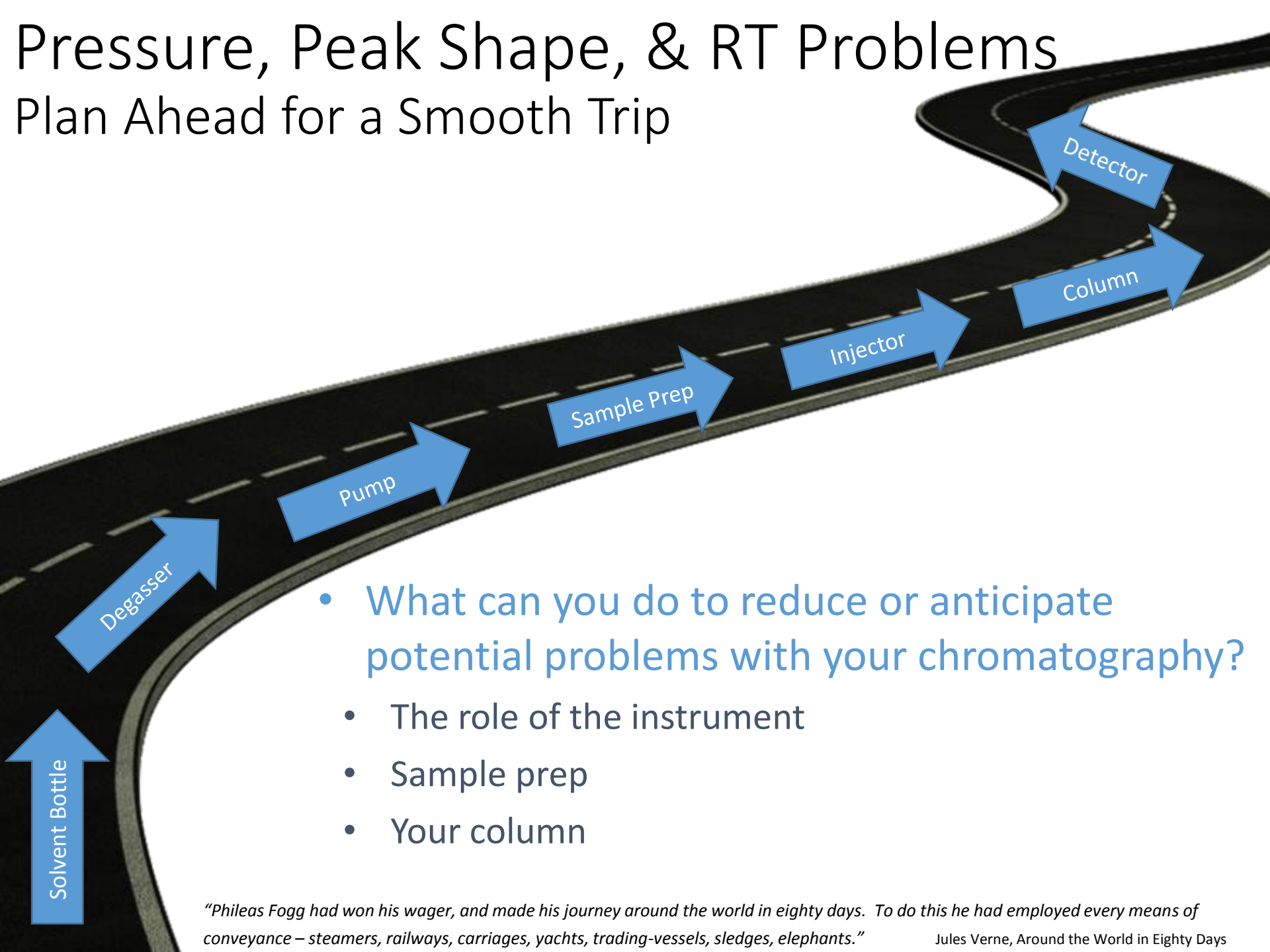
Around the LC System in 45 Slides

Rita Steed and Alex Ucci

Pressure, Peak Shape, & RT Problems

Plan Ahead for a Smooth Trip



- What can you do to reduce or anticipate potential problems with your chromatography?
  - The role of the instrument
  - Sample prep
  - Your column

"Phileas Fogg had won his wager, and made his journey around the world in eighty days. To do this he had employed every means of conveyance – steamers, railways, carriages, yachts, trading-vessels, sledges, elephants." Jules Verne, Around the World in Eighty Days

What's in Your Solvent Bottle?

Mobile Phase

- Use only high quality HPLC or MS grade solvents
  - Do not filter
- Filter buffer and salt solutions
  - Filter porosity: 0.45 or 0.2 µm
  - Make sure the filter material is compatible
- Avoid algae/microbial growth
  - Frequently replace the mobile phase with a clean bottle
  - Adding some organic to aqueous mobile phases can inhibit growth
  - Consider avoiding light exposure

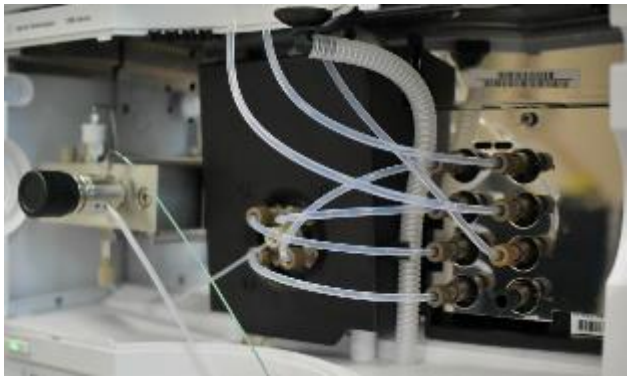
- ➢ Can cause degasser problems
- ➢ Can be a source of ghost peaks

Solvent inlet filter

- Not a replacement for good mobile phase hygiene
- Glass solvent inlet filter (20 µm), 5041-2168
- Stainless steel solvent inlet filter, 01018-60028
  - Stainless inlet filter recommended for LCMS

Degasser

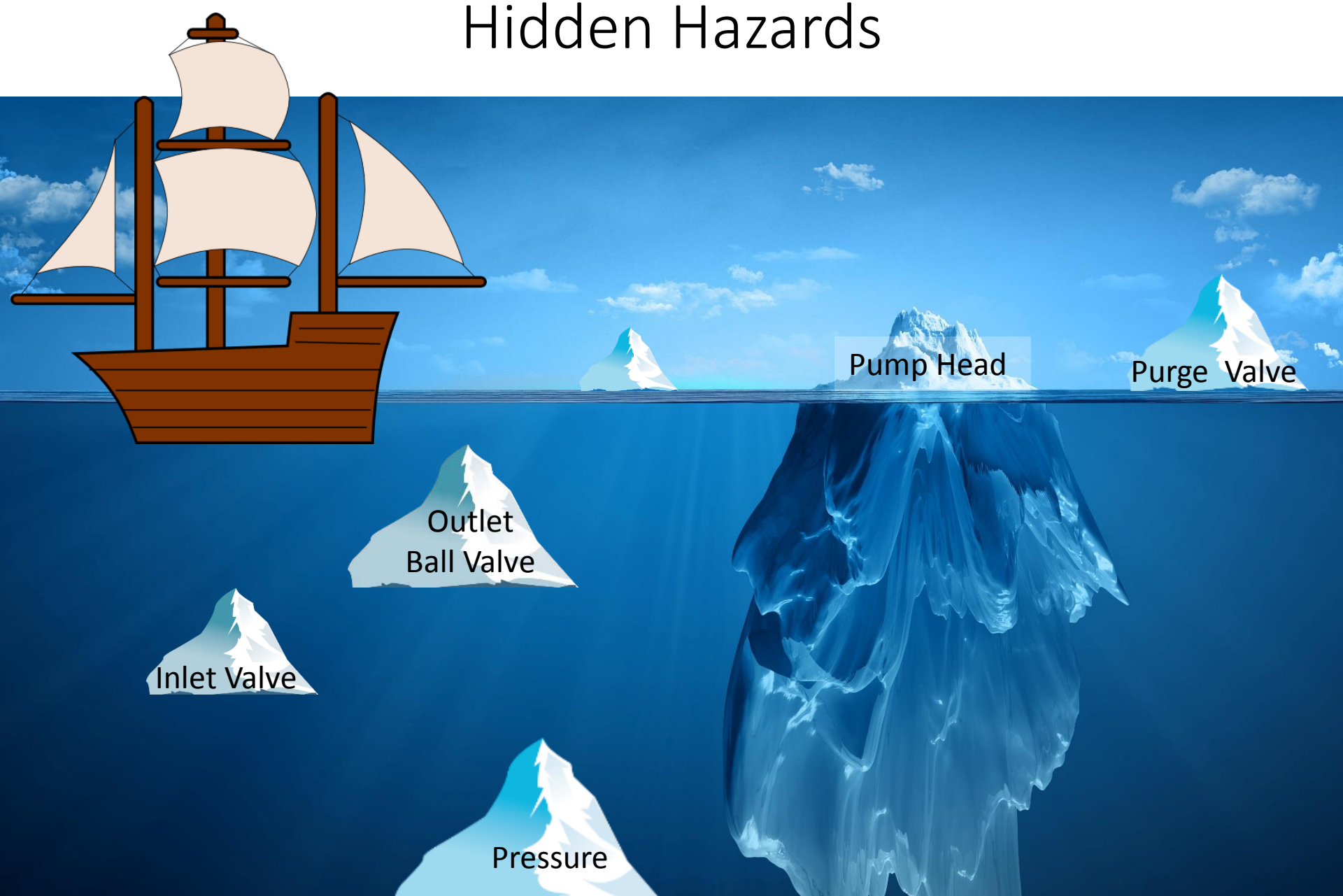
Degasser Care



- Check for bubbles in outlet lines
- Avoid blockages by flushing out buffer salts with water when changing mobile phases
- When switching solvents, make sure they are miscible
- Do not leave the degasser for an extended period of time with aqueous mobile phase to avoid microbial growth
- Unused channels should be flushed with water to remove any buffers, and left in organic solvent

Pump

Pump Care
Hidden Hazards



Pump Care –

Inlet Valve

- Pressure ripple unstable; Active inlet valve cartridge may be dirty
- Inlet valve issues can lead to
  - poor pump performance
  - detector baseline noise
  - unstable system pressure
  - poor retention-time precision

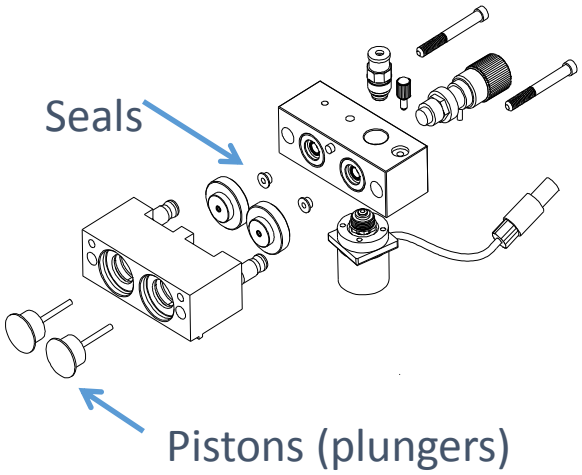
Outlet Ball Valve

- A failing outlet ball valve causes backflow of solvent
  - poor pump performance
  - detector baseline noise
  - unstable system pressure (pressure ripple)
  - poor retention-time precision
- Outlet valves on older Agilent LC's have a separate gold seal cap, which can still be replaced
- However, the current valve design has an integrated gold seal

5067-4728

Pump Care –
Pump Head

- Perform seal wear-in procedure after installation of black reversed-phase seals
- Replace on a regular basis, before there is a problem
- Set up a replacement schedule for your instrument based on usage and mobile phase composition
- Leaking pump seals can lead to
  - poor pump performance
  - unstable system pressure (pressure ripple)
  - Detector baseline noise
  - poor retention-time precision



0905-1420
Polyethylene (NP)
5063-6589, PTFE (RP)
0905-1719, for 1290

5064-8211

Purge Valve

- Dirty frit in the purge valve often a source of high pressure
- A pressure drop of >10 bar across the frit (5 mL/min water with purge valve open) could indicate a blockage
- Change after changing pump seals and seal wear-in

Seal cap
5067-4728

PTFE frits
01018-22707

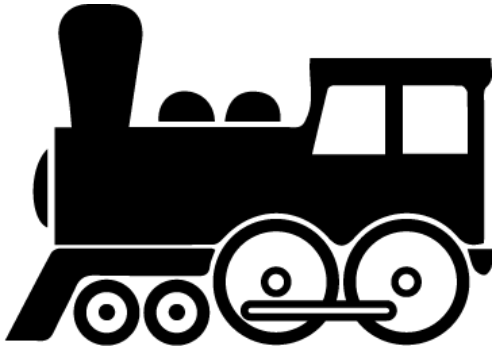
Pressure

What causes high pressure?

- **Particulates in mobile phase**
  - Improperly filtered buffer solutions
  - Buffer precipitation
  - Microbial growth (see appendix for more info)
  - Seal debris
- **Particulates in the sample**
  - Precipitated sample
  - Insoluble matrix components

Common blockage points

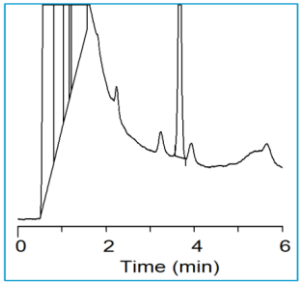
- Purge valve frit
- Autosampler needle/needle seat
- Column frit



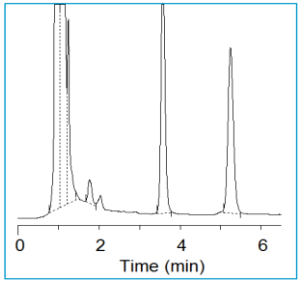
Why perform Sample Preparation?

- To acquire desired sensitivity/selectivity
- To reduce contamination/carryover issues
- Use of sensitive and expensive instruments:
  Protect your investment!!!

Pesticides in Avocado *without* SP



Pesticides in Avocado with SP



Common Tools to Help you Along your Journey

- **Solid Phase Extraction (SPE)**
- Multi-step approach for highest level of sample cleanup
- **QuEChERS (dSPE)**
- Sample cleanup by extraction of bulk interferences
- **Captiva ND Lipids (PPT and lipid removal)**
- Removes precipitated proteins by in-well protein precipitation and also removes phospholipids
- **Filtration**
- Simple and fast removal of particulates

Cleanliness

Selectivity

Complexity

Cost

Captiva Filtration and it's Benefits

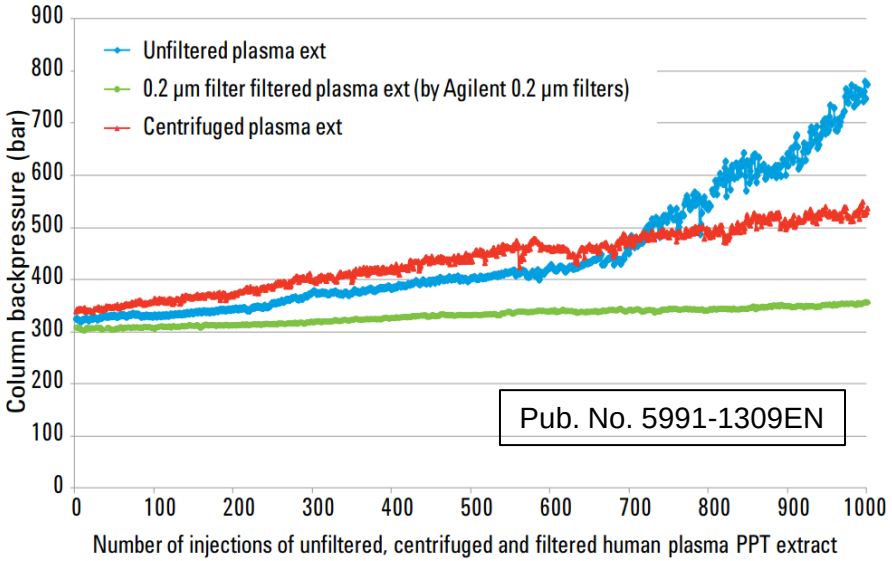
Filtration is basic sample preparation method for all kinds of samples

Physically removes particulates from the sample

Prevents blocking of capillaries, frits, and column inlet (especially for UHPLC)

Results in less downtime of the instrument for repairs

Results in less wear and tear on the critical moving parts of the injection valves



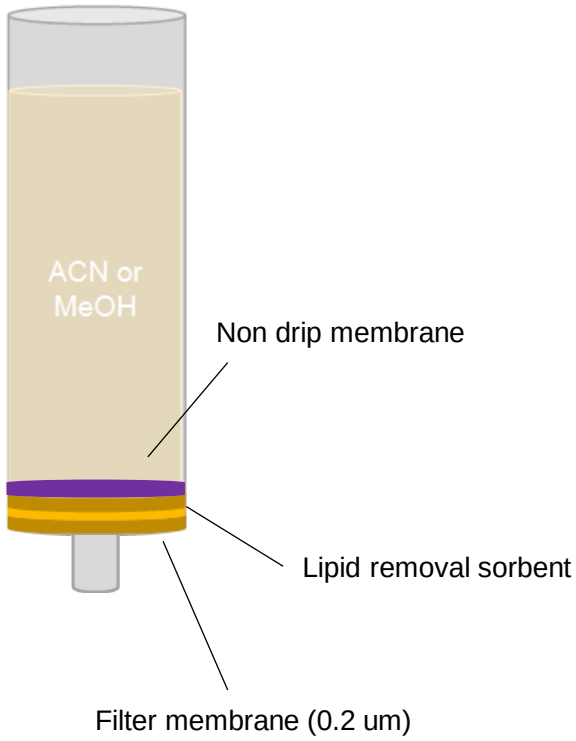
Unfiltered, centrifuged, and filtered plasma extracts
Zorbax RRHD Eclipse Plus C18, 2.1 x 50 mm, 1.8 µm column, PN 959757-902

Captiva Syringe Filters Guide 5991-1230EN

Syringe Filter Selection Tool

Functionalized Filtration: Captiva ND + Captiva ND **Lipids**

Captiva ND Lipids



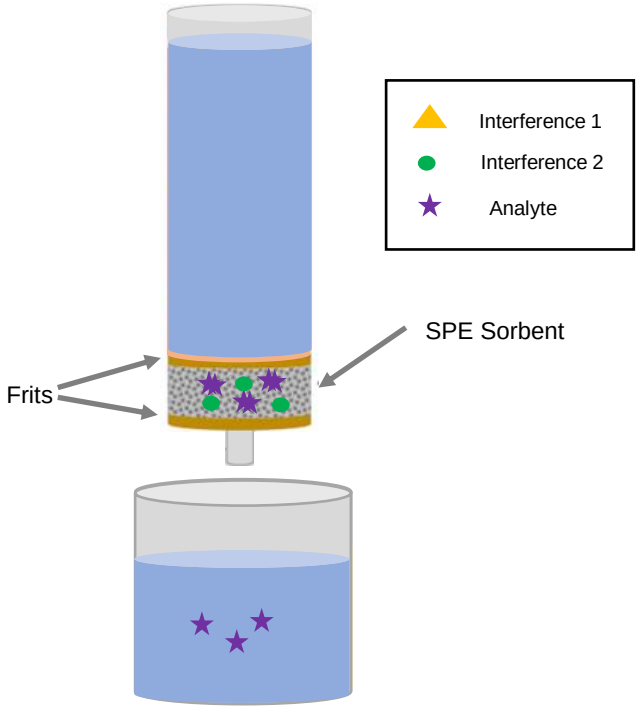
- If you have a sample with excess proteins and/or lipids, these can also cause problems
- Captiva ND/Captiva ND **Lipids**- easy to use products available in both cartridges and 96-well plate formats
- Both contain a non-drip (ND) membrane allowing for in-well protein precipitation
- Captiva ND **Lipids**-Contains an extra lipid removal sorbent to remove lipids
  - 3 in 1 benefit of filtering, removing proteins, and lipids
  - Use of Captiva ND Lipids will reduce ion suppression, increase analyte sensitivity and detection, and extend the lifetime of your analytical column

ACN = acetonitrile / MeOH = methanol

Captiva Portfolio Overview 5991-5039EN

What is Solid Phase Extraction?

- Uses a plastic disposable cartridge packed with varying amounts of sorbent between two frits
- Sorbent can be silica/polymeric based and involve a variety of phases
  - chosen based on the chemistry of the analyte of interest and interferences
- Uses of SPE: removal of interferences, concentration/enrichment, desalting, solvent exchange



Typical SPE Sequence

Step 1: Condition the cartridge

Step 2: Apply sample

Step 3: First wash of the cartridge (interference removal)

Step 4: Apply solvent to elute

Why

Choose

SPE?

- Flexible - match a broad spectrum of sample and target compound types to different sorbents and forms
- Wide array of formats and sorbents for lower detection limits and longer instrument uptime from cleaner extracts
  - Agilent has over 40 sorbent materials/phases available in the Bond Elut line of products!
- Increase sample throughput with automation-friendly formats
- Easy adoption of methods due to high number of publications and applications
- Best balance of sample cleanliness, accuracy of results, and cost-per-sample

QuEChERS

Easy

Effective

Safe

Quick

Cheap

Rugged

- Screening of pesticide residues in fruit and vegetables
  - Developed to make sample cleanup of food faster, simpler, less expensive, and greener
- Now used with other matrices and compound classes as well

Consists of two steps, and thus **2 kits**:

Step 1: Liquid Extraction

Step 2: Dispersive SPE / Interference Removal

Bond Elut Dispersive SPE Kits

Dispersive kit contains:

Centrifuge tubes containing pre-weighed SPE sorbents such as:

C18: removes residual fats and lipids

PSA: 'primary/secondary amine' for removal of organic acids and sugars

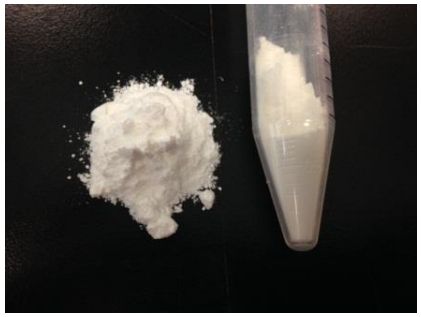
GCB: graphitized carbon black, removes pigments

EMR-Lipid: removes unbranched hydrocarbon chains (lipids)

- Kits available for different food types
- For both AOAC (US) method and EN (Europe)
- QUECHERS is a non-selective technique, does not remove ALL the matrix, but just enough
- SPE sorbent also available as bulk material

New dSPE sorbent!: EMR-Lipid

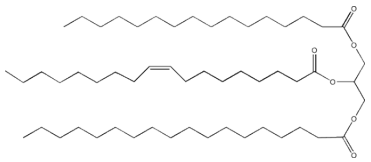
Enhanced Matrix Removal: EMR-Lipids



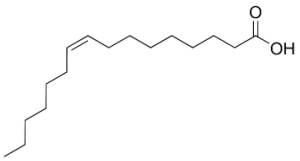
1.0 g EMR in 15 mL tube

When "activated" by water…

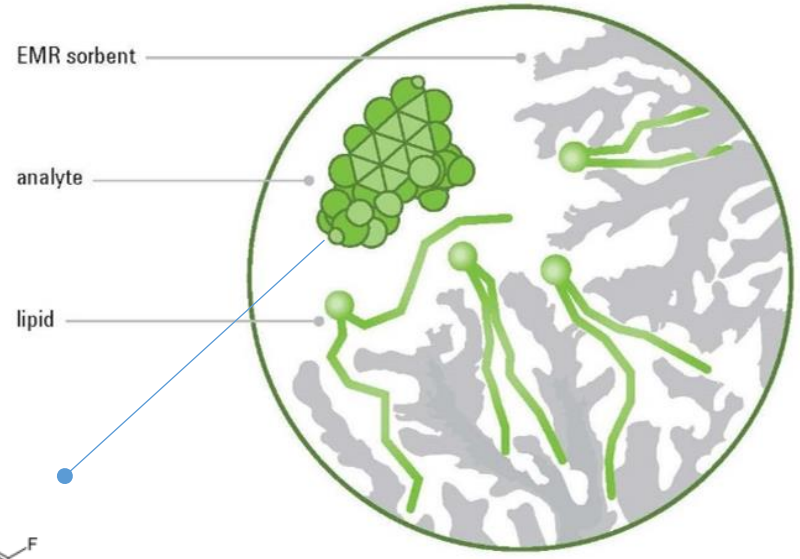
- The materials selective hydrophobic interactions increase.
- Suspension of nano particles (high surface area).
- Rapidly interacts with straight chain, "lipid-like" functional groups.
- Does not retain analytes



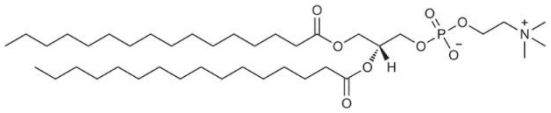
Triglycerides

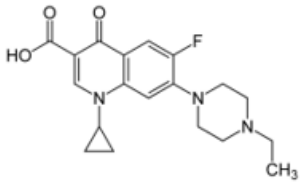


Free Fatty Acids



Phospholipids

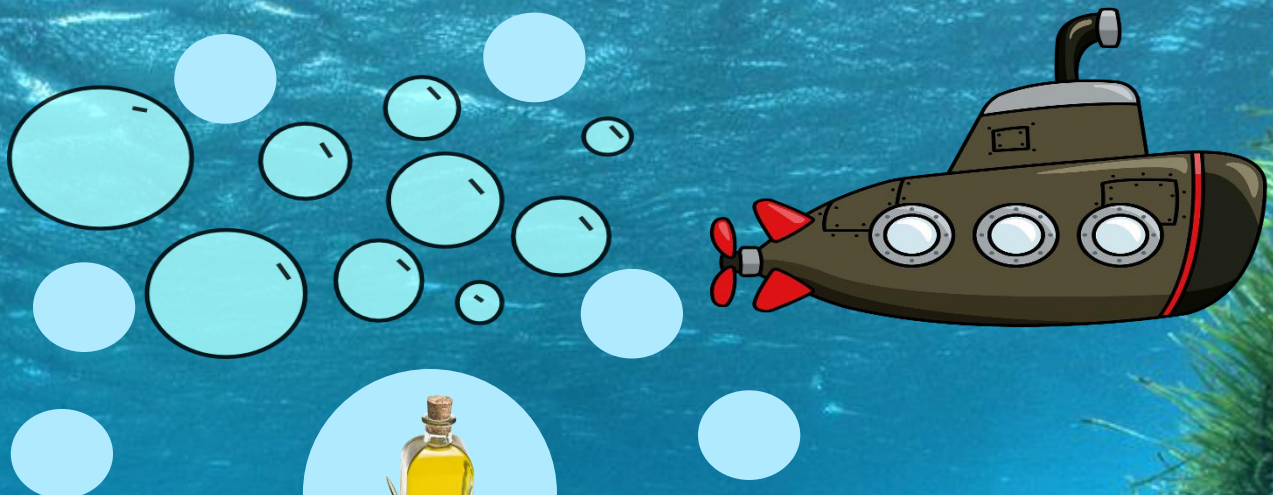




Fluoroquinolones

EMR-Lipid Mechanism –
Combined size exclusion and hydrophobic interaction.

EMR-Lipid Product Offering



Fits into existing workflows:

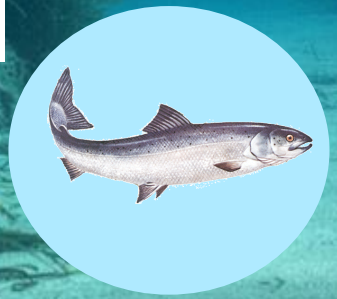
- after QUECHERS extraction
- after Liquid Extraction

Protocols also require a “polish” step after EMR to remove water and dissolved solids before injection

Effective Removal of lipids (fats)

Tubes containing 1g of EMR sorbent

5982-1010



Polish Tube

$NaCl/MgSO_4$

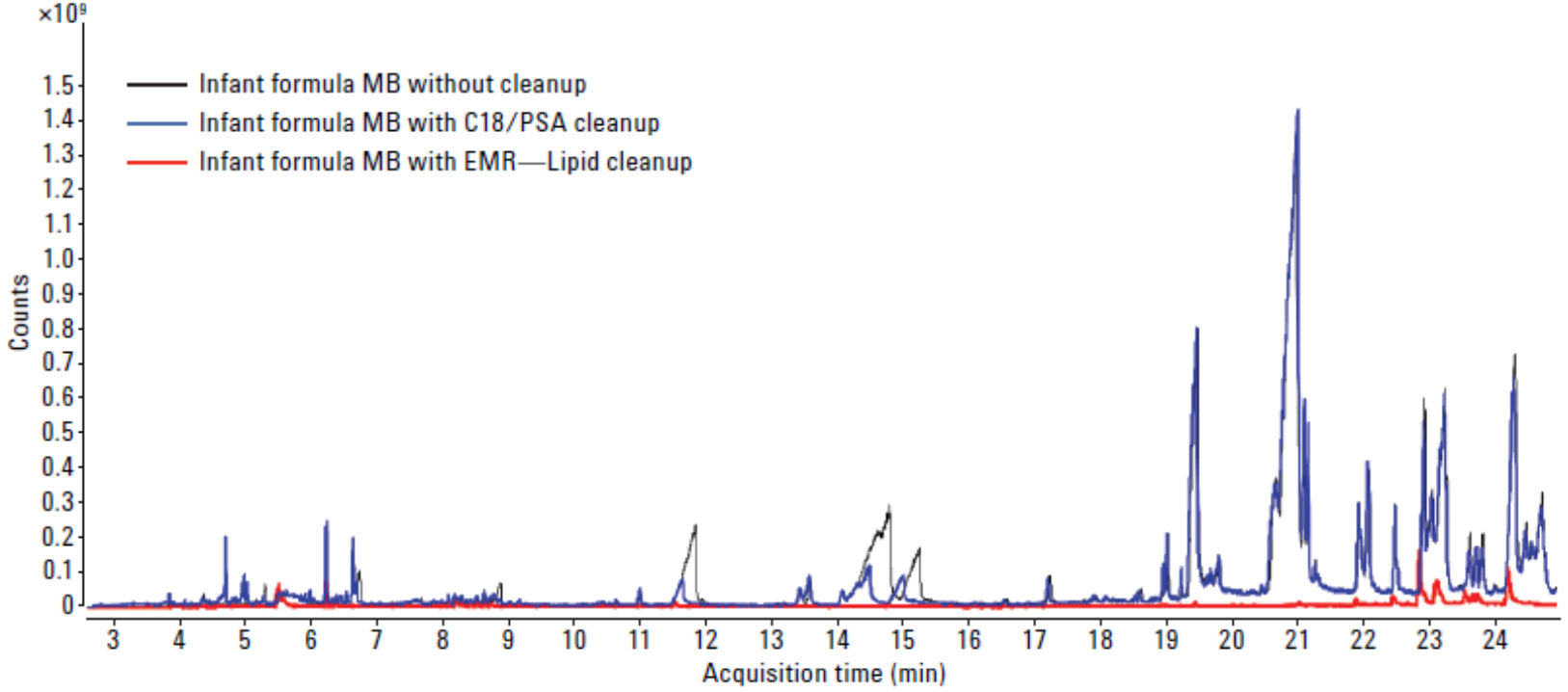
(p/n 5982-0101)

Polish Pouch

$MgSO_4$

(p/n 5982-0102)

Aflatoxin Analysis in Infant Formula (5991-6818EN)



A Poroshell 120 SB-C18 column was used to separate aflatoxin compounds in plasma.

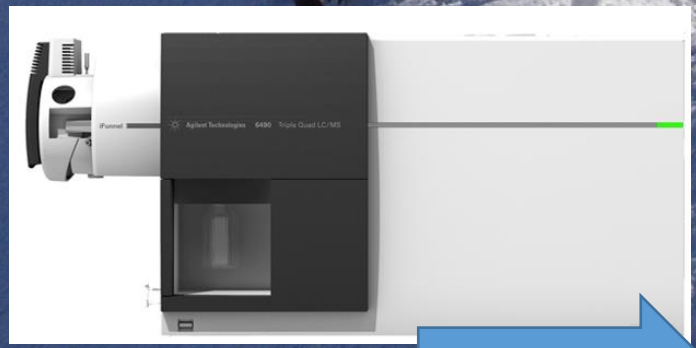
A full scan was run of infant formula matrix blank without cleanup, with traditional QUECHERS with C18/PSA dSPE, and using EMR-Lipid.

EMR-L incorporated into QUECHERS offers SPE cleanliness with the simplicity of the QUECHERS methodology and works especially well with high lipid matrices.

Productivity Benefits with Sample Preparation

More Matrix Removal = Less Matrix Entering System = Time and Cost Savings!

- ✓ Less matrix build-up
  - Less interferences
  - Improved S/N
  - Better reproducibility
- ✓ Better chromatography
  - Less time spent on data analysis/manual integration
  - Less time spent on re-runs/recalibrations
- ✓ Less maintenance
  - Less instrument down-time
  - Saves $$ on consumables/services
- ✓ Less troubleshooting
  - "Is it my column or my MS"?
  - Less instrument down-time

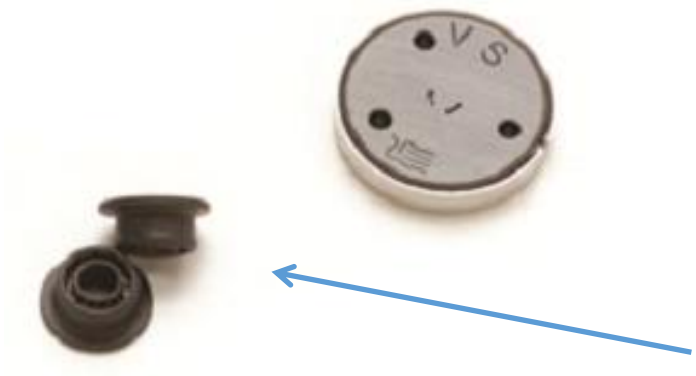


Injector

Autosampler Care

Five main maintenance points:

- ✓ Needle
- ✓ Loop capillary
- ✓ Needle seat
- ✓ Injection valve rotor seal
- ✓ Metering device seal

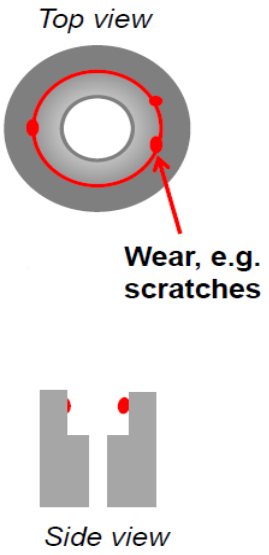


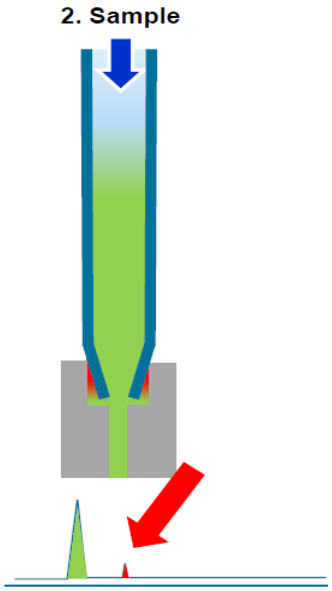
- Rotor seal wear is often a cause of sample carry-over
- Exchange the rotor seal
  - After approximately 30000 to 40000 injections, or sooner depending on your specific sample and mobile phase
  - When injection reproducibility or leakage indicates wear
- Exchange the metering seal when autosampler reproducibility indicates seal wear

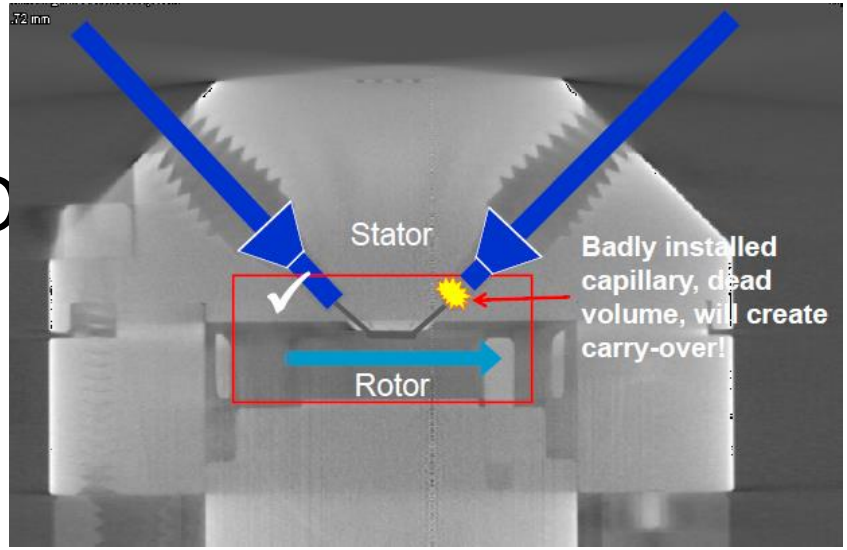
Autosampler Carryover

Common sources

- Exterior of needle (use needle wash)
- Worn needle seat
- Worn rotor seal
- Poorly made fitting





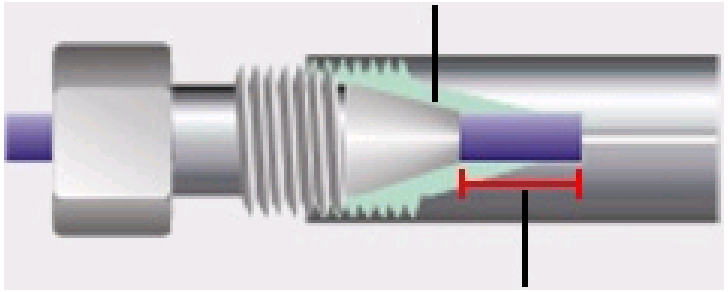



Connections
Too Long
InfinityLab
Too Short
Quick Turn
Quick Connect

Poorly Made Connections

Wrong … too long

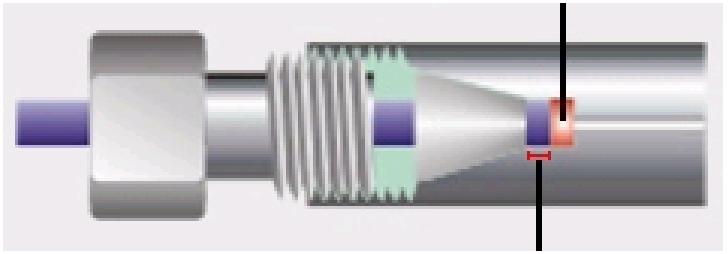
Ferrule cannot seat properly



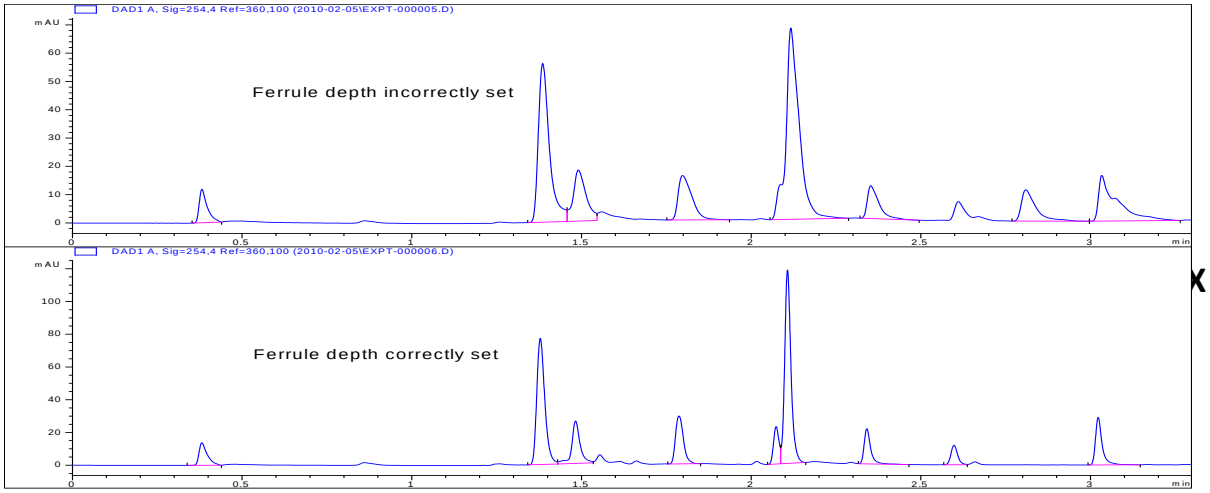
If Dimension X is too long, leaks will occur

Wrong … too short

Mixing Chamber



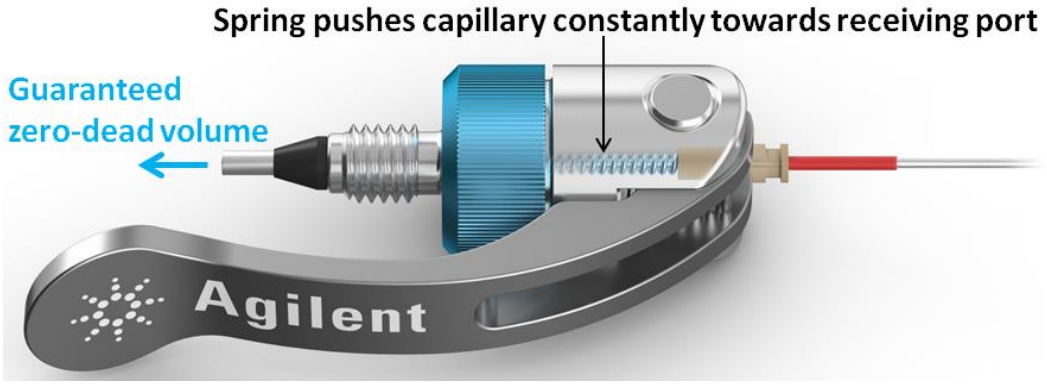
If Dimension X is too short, a dead-volume, or mixing chamber, will occur



Column : 50mm x 2.1mm x 1.8um Eclipse Plus C18

Simplify Connections

InfinityLab Quick Connect & Quick Turn



Stem length is adjustable through the spring

Quick Turn fitting

Ease of use

- Quick Connect
  - Seals with a simple turn and lever
    - Spring-loaded design=reproducible connection
    - No dead volume
    - Consistent chromatographic performance
    - Finger-tight to 1300 bar
- Quick Turn
  - Stem length is adjustable through the spring
    - Compatible with all types of LC columns
    - Finger-tight to 600bar, wrench-tight to1300bar

Filtration – Keep the Flow Going

What causes high pressure?

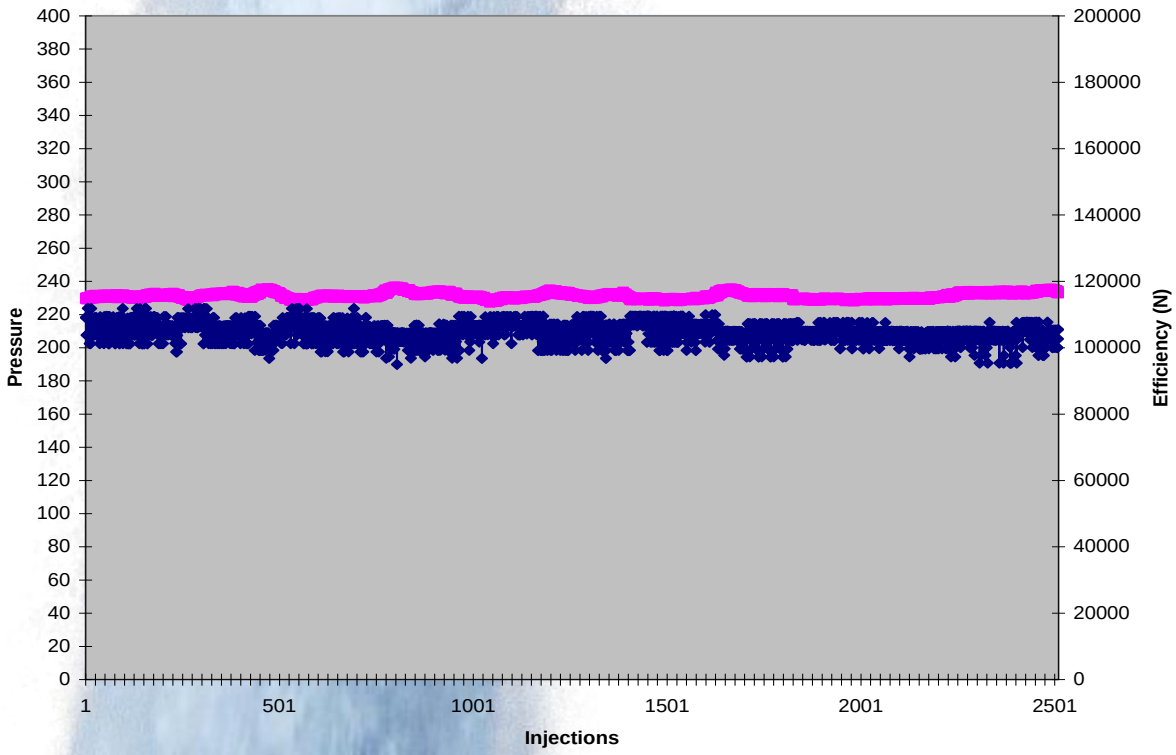
- **Particulates in mobile phase**
  - Improperly filtered buffer solutions
  - Buffer precipitation
  - Microbial growth
  - Seal debris
- **Particulates in the sample**
  - Precipitated sample
  - Insoluble matrix components

Common blockage points

- Purge valve frit
- Autosampler needle/needle seat
- Column frit

Poroshell 120 with 2um frit Resists Plugging

Column: Poroshell 120 EC-C18, 3.0 x 50mm, 2.7um LC: Agilent 1200 RRLC (SL)
Sample: Precipitated Plasma: 2 parts Plasma: 7 Parts 20/80 Water-MeCN w/0.1 % Formic Acid with 1 Part Diflusinal in 50/50 Water-MeCN 10 ug/ml (Final concentration Diflusinal 1 ug/ml) Shaken and allowed to settle 10 minutes
Not Centrifuged/ Not Filtered Diflusinal in Plasma
Injection Volume: 1ul injections



Solvent A: Water w/0.1 % TFA
Solvent B: MeCN w/0.08 % TFA
Flow Rate 1 ml/min 1 ul injection

Time	% B
0	20
0.5	90
0.6	90
1.1	20
2.5	20

Long Lifetime = reduced column costs

In-Line Filters & Guards

In-line filters

- Help extend column lifetime
- Not intended to be a replacement for good sample cleanup

RRLC in-line filter, 0.2 µm filter
4.6 mm ID, 5067-1553
2.1 mm ID, 5067-1551

1290 Infinity in-line filter,
0.3 µm, 1200 bar, 5067-4638

Guards

- Install a guard column with dirtier samples
  - Can extend life of column
  - Utilize more inexpensive guard columns rather than column replacements
- Types
  - Cartridge
  - 600 & 1200 bar Fast Guards

Select the Best Column

Low pH

High pH

Stability

Secondary Interactions

Retention

Choose Suitable Column Phase

Mobile Phase pH →

Acidic

StableBond, pH 1 – 4

SB can use temperatures up to 100°C

Intermediate

Poroshell EC/Eclipse Plus, pH 2 – 9

Poroshell EC & Eclipse Plus for mid-pH applications

Basic

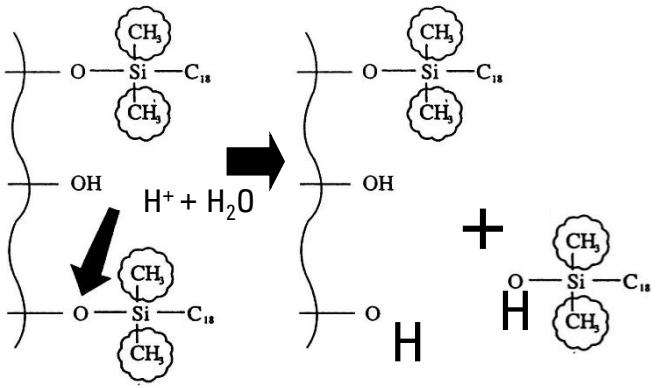
HpH, pH 8 – 11.5

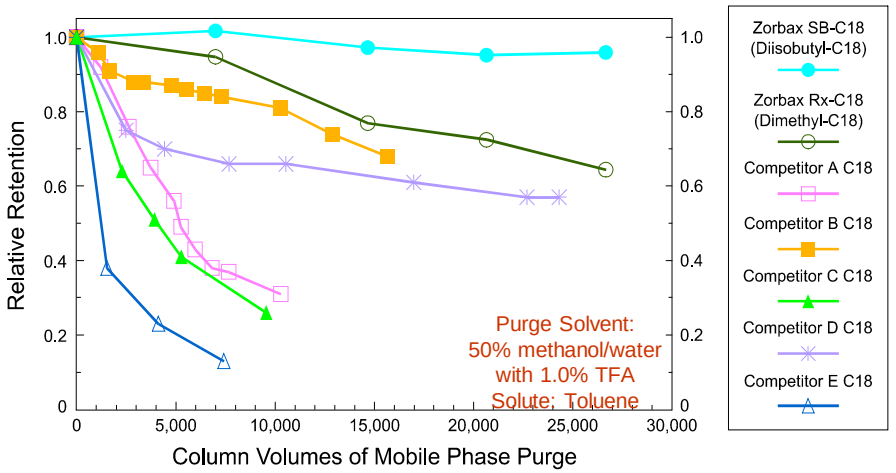
HpH C8 & C18 for high pH

Silica-based HPLC Mechanisms of Degradation

Low pH (pH <3)

- Hydronium-catalyzed hydrolysis of bonded phase siloxane
  - Loss of Bonded Phase
  - Change in retention times (usually decrease)



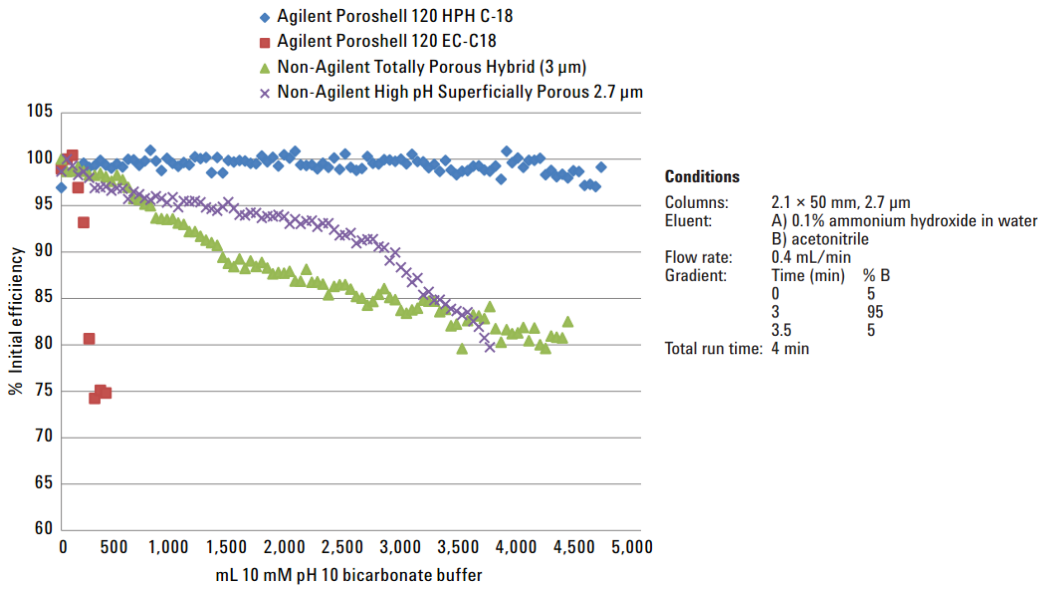


Accelerated degradation tests of C18 bonded phases at low pH and high temperatures (pH 0.8, 90°C)

Silica-based HPLC Mechanisms of Degradation

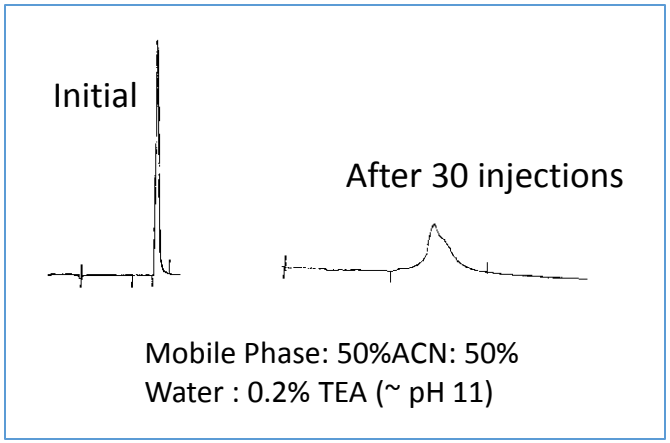
Intermediate to High pH(pH >6-7)

- Dissolution of the underlying silica by the hydroxide ion
  - Loss of silica, void development
  - Loss of resolution



Conditions

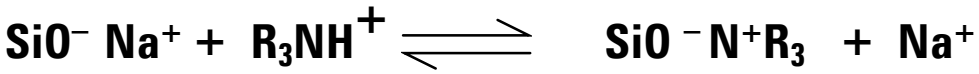
Columns:	2.1 × 50 mm, 2.7 µm
Eluent:	A) 0.1% ammonium hydroxide in water B) acetonitrile
Flow rate:	0.4 mL/min
Gradient:	Time (min) % B 0 5 3 95 3.5 5
Total run time:	4 min



Mobile Phase: 50%ACN: 50%
Water : 0.2% TEA (~ pH 11)

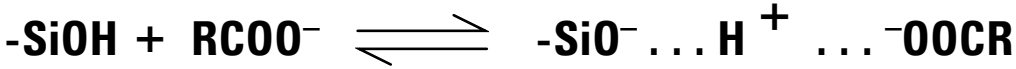
Potential Secondary Interactions

Ion-exchange



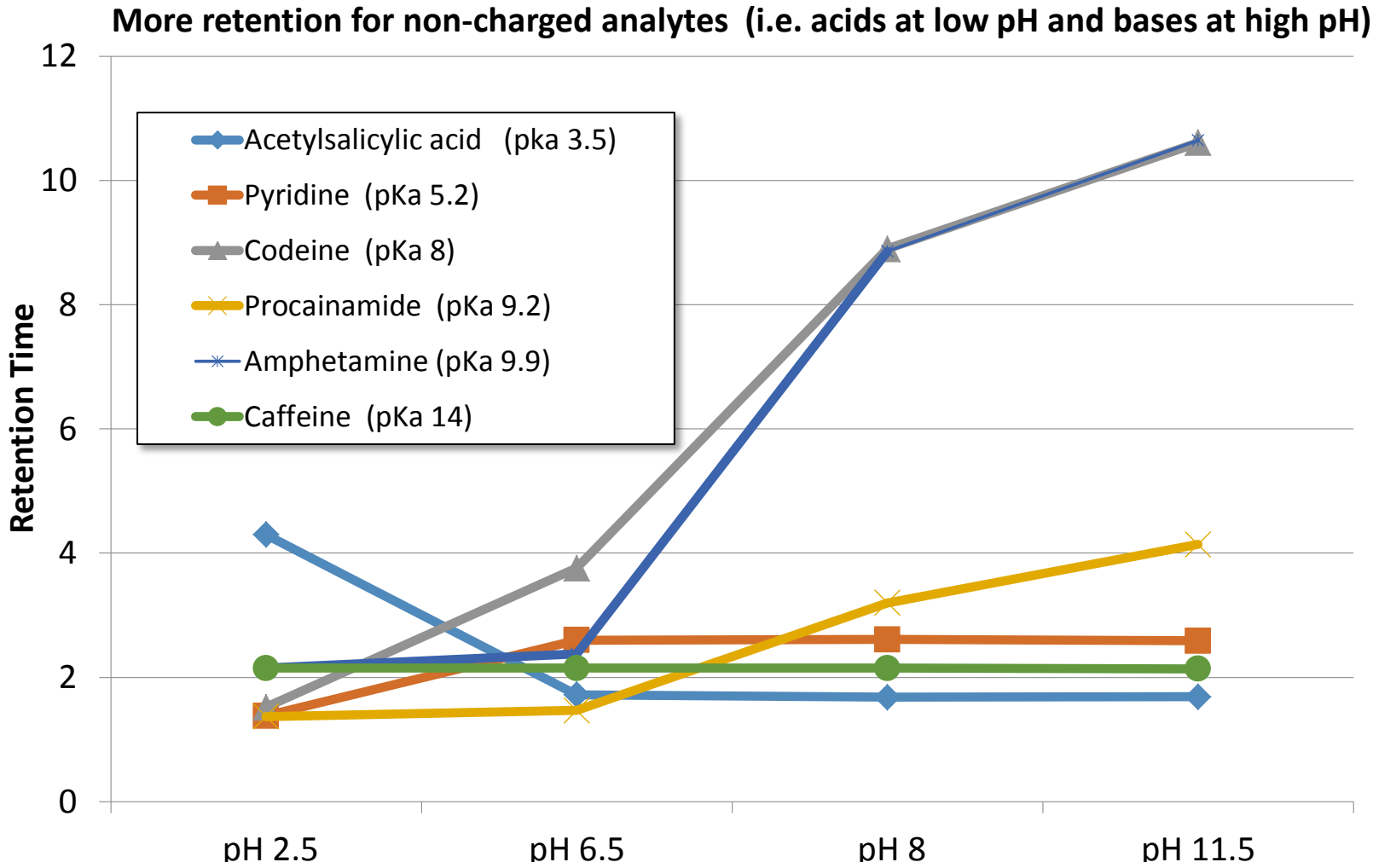
1. Ionized silanols (SiO^-) will ion-exchange with protonated bases (R_3NH^+) which can cause tailing and method variability. This occurs most often at mid pH where silanols are ionized.

Hydrogen bonding



2. Unprotonated acids can compete for H^+ with protonated silanols. This can occur at low pH.

Ionizable Compounds
pH Related RT Changes are Compound-Dependent



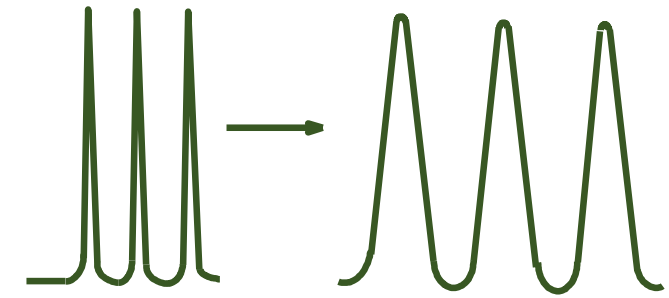
Mobile Phase: 45% MeOH, 55% 20 mM Phosphate Buffer

Retention Time Shifts

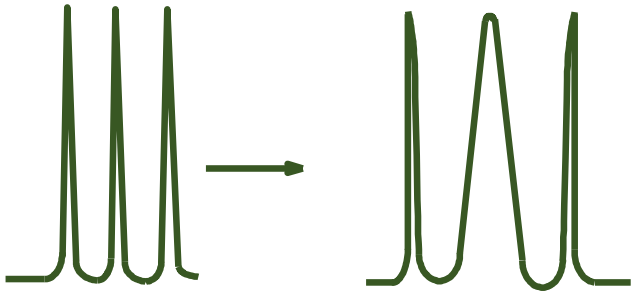
- All Peaks Shift to <Retention (acids, bases, neutrals)
  - Loss of Bonded-Phase
  - Mobile Phase Unstable (less likely)
  - Solvent Delivery System (flow rate)
- All Peaks Shift to >Retention
  - Loss of Organic Solvent in Aqueous / Organic Mix
  - Column Change (less likely)
  - Solvent Delivery System (flow rate)
- Ionic Peaks Shift Retention
  - Loss of Volatile Mobile Phase Component (ionic strength, pH shift)
  - Column Change (bonded phase or contamination)

Peak Shape

Peak Shape
Broad Peaks



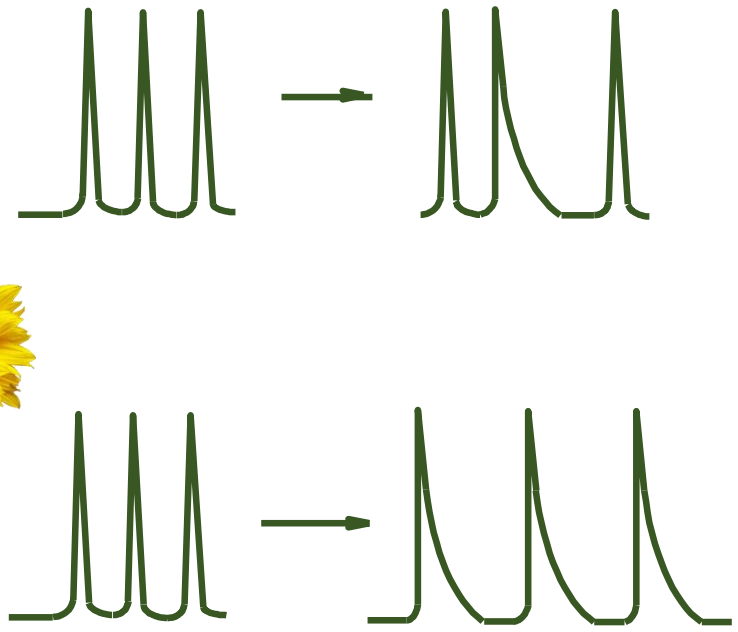
- **All Peaks Broadened**
- Loss of column efficiency - column void
- Large injection volume/mass
- High viscosity mobile phase
- Sample solvent mismatch



- **Some Peaks Broadened**
- Late elution from previous sample
- High MW sample – protein or polymer

Peak Shape

Tailing Peaks, Symmetry >1.5



- **Some Peaks Tail**
- Secondary retention effects
  - Residual silanol interactions
- Small Peak eluting on tail of larger peak

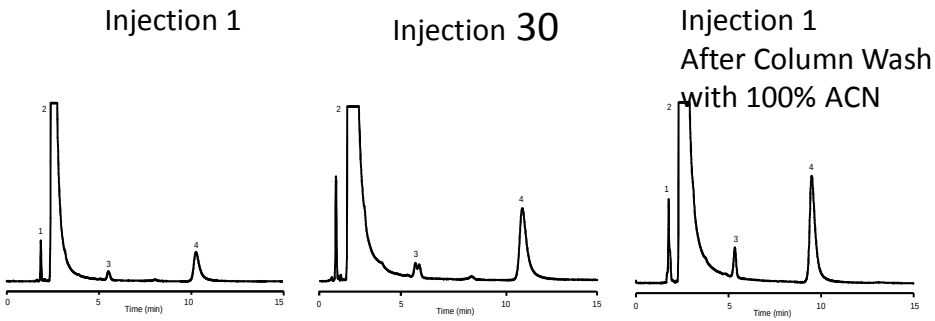
- **All Peaks Tail**
- Extra-column effects
- Bad columns
- Contamination on column inlet
- Metals
- Inappropriate sample size/solvent

Peak Shape
Split and Double Peaks

Can be caused by:

- Column contamination
- Only 1 peak a doublet
  - Co-eluting compounds
- Partially plugged frit
- Column void
- Injection solvent effects

Column: StableBond SB-C8, 4.6 x 150 mm, 5 mm Mobile Phase: 60% 25 mM Na_2HPO_4, pH 3.0 : 40% MeOH Flow Rate: 1.0 mL/min
Temperature: 35°C Detection: UV 254 nm Sample: Filtered OTC Cold Medication: 1. Pseudoephedrine 2. APAP 3. Unknown 4. Chlorpheniramine



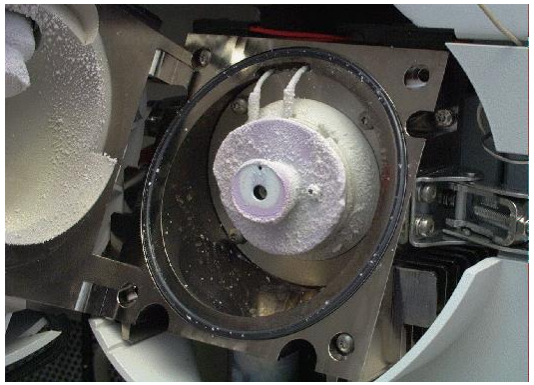
Detector Care
UV Detectors

DAD tungsten lamp

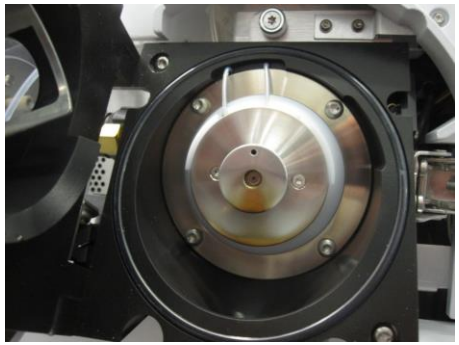
- Two types
  - VWD
  - DAD/MWD
- Simple Maintenance
  - Lamp replacement
  - Flow cell cleaning or replacement
- Keep in mind the pressure rating of your flow cell – another detector fraction collector in the flow path will increase the backpressure on the flow cell
- Avoid using flow cells with quartz windows at pH 9.5 or greater
- Make sure the flow cell contains 5 or 10% organic to prevent microbial growth when not in use
- Avoid leaving buffer solutions in the flow cell which can crystallize

Detector Care

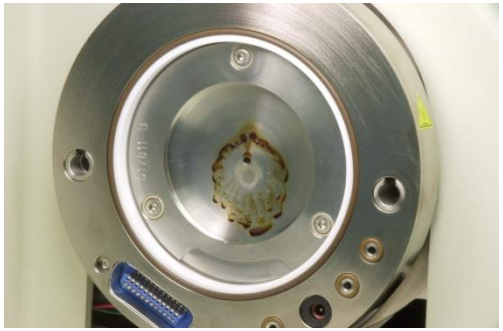
Examples of Instrument Contamination/Road Blocks



Salt build-up in LC-MS ion source from unextracted salts



ESI Ion Source contamination after 3000x Urine Dilute/Shoot Injections



Curtain plate after injection of 25 samples with extractions from raisins without cleanup

Summary

- You can reduce or prevent problems by thinking ahead
- Some instrument parts should be replaced on a regular basis, before there is a problem
- Develop a maintenance routine that works for you (see appendix)
- Sample preparation is a powerful tool in addressing common chromatography and mass spectrometry challenges
- Sample preparation is an investment that can help solve challenges and achieve your analytical goals
- Make sure you choose the best column for your sample and conditions

Technical Support

- ***Still have questions?***

- 800-227-9770 (US & Canada)
- Options 3, 3, 2 (LC columns)
- Options 3, 3, 3 (SPP)
- Email: lc-column-support@agilent.com
- SPP-Support@agilent.com
- www.agilent.com/chem