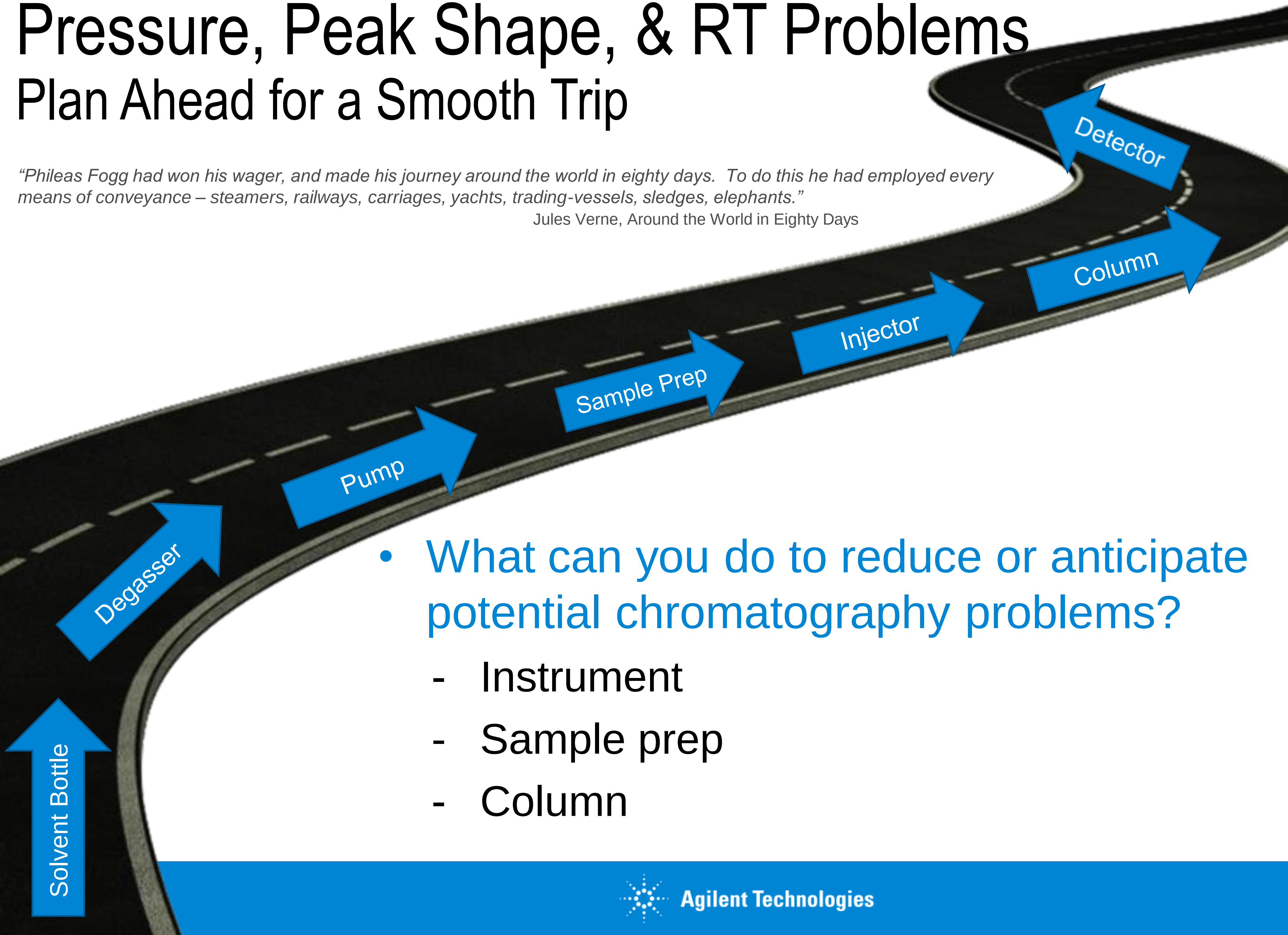
Around the LC System in 45 Slides

Rita Steed and Golnar Javadi

Pressure, Peak Shape, & RT Problems
Plan Ahead for a Smooth Trip

> *“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 can you do to reduce or anticipate potential chromatography problems?
  - Instrument
  - Sample prep
  - Column

What's in Your Solvent Bottle?

Mobile Phase

- Use <u>only</u> 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)

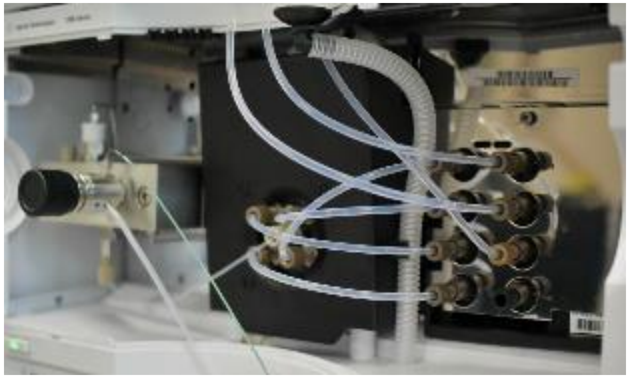
5041-2168

Stainless steel solvent inlet filter, 01018-60028

- Stainless inlet filter recommended for LCMS

Degasser

Degasser Care

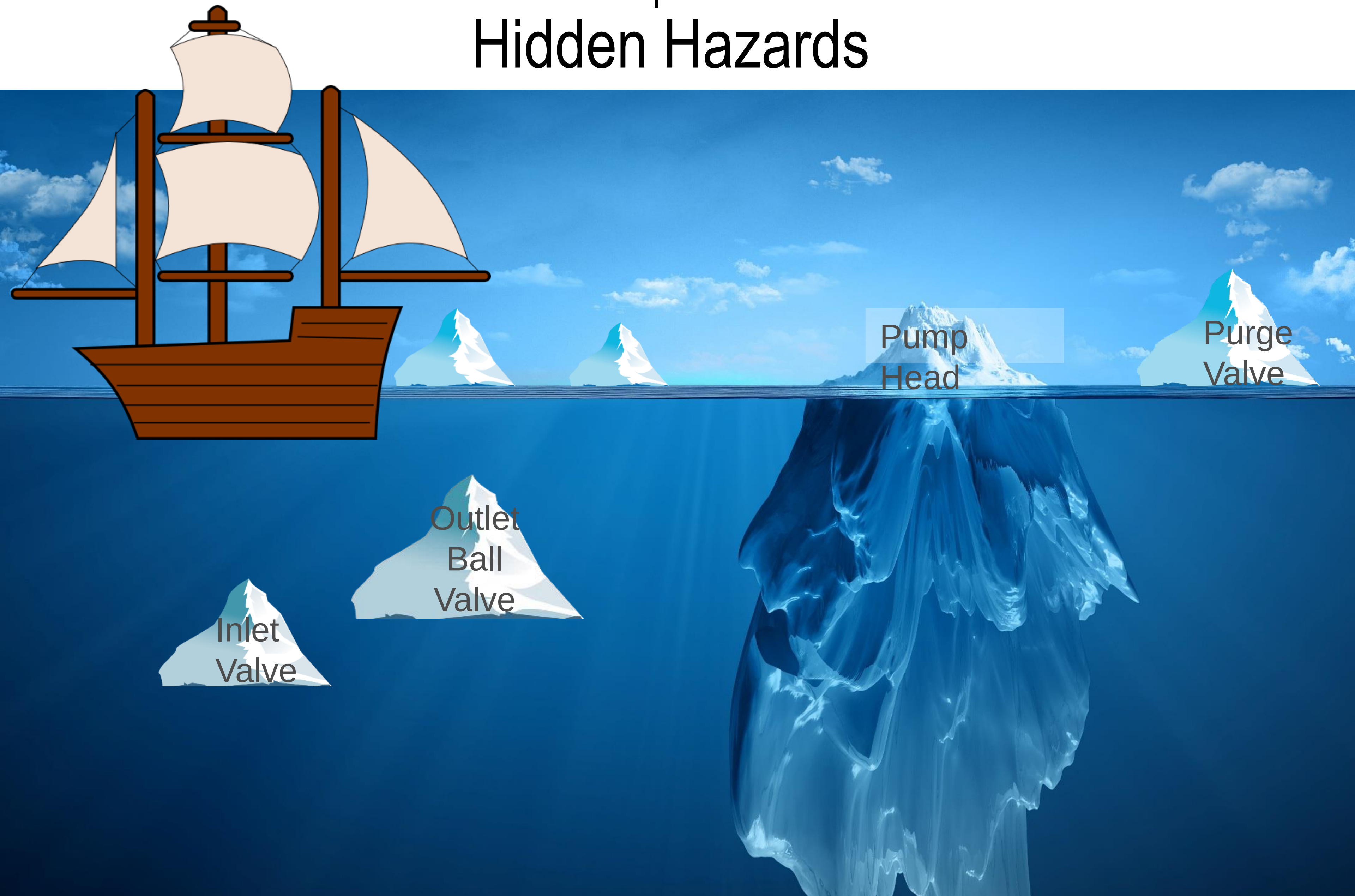


- Check for bubbles in outlet lines
- Avoid blockages by flushing out buffer salts with water when changing mobile phases
- When switching solvents, make sure they are miscible
- Do not leave degasser for an extended period of time with aqueous mobile phase - avoids microbial growth
- Flush unused channels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

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

Pump Care – 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

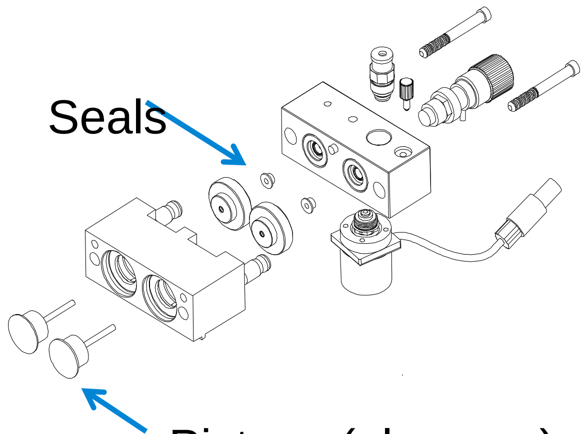
- Current valve design has an integrated gold seal

5067-4728

Pump Care –

Pump Head

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




Agilent Technologies

HPLC Maintenance Videos

Changing the Seals in a 1260 Bianary, Quaternary, or Isocratic Pump without Seal Wash Option

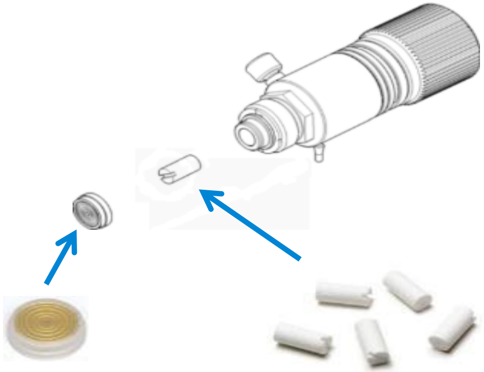
https://www.youtube.com/watch?v=vFUVHssMnx4

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

5064-8211

Pump Care – Purge Valve

- Dirty frit in purge valve - often 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

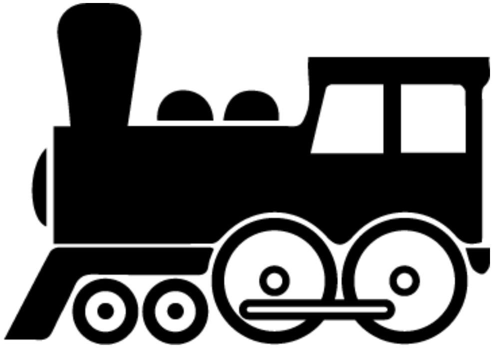
Common blockage points

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

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

Sample Prep



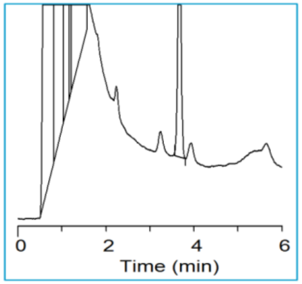
Why perform Sample Clean-Up?

To acquire desired sensitivity/selectivity

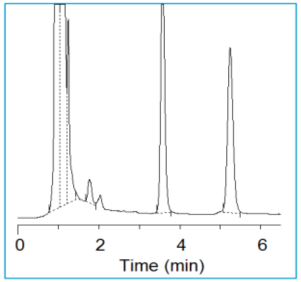
To reduce contamination/carryover issues

Use of sensitive and expensive instruments: ***Protect your investment!!!***

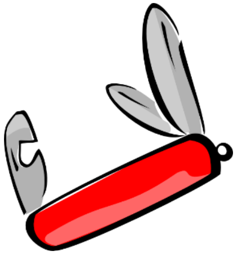
Pesticides in Avocado *without* clean-up



Pesticides in Avocado with clean-up



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 EMR-Lipid (PPT and lipid removal)

Removes precipitated proteins by in-well protein precipitation and also removes lipids

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

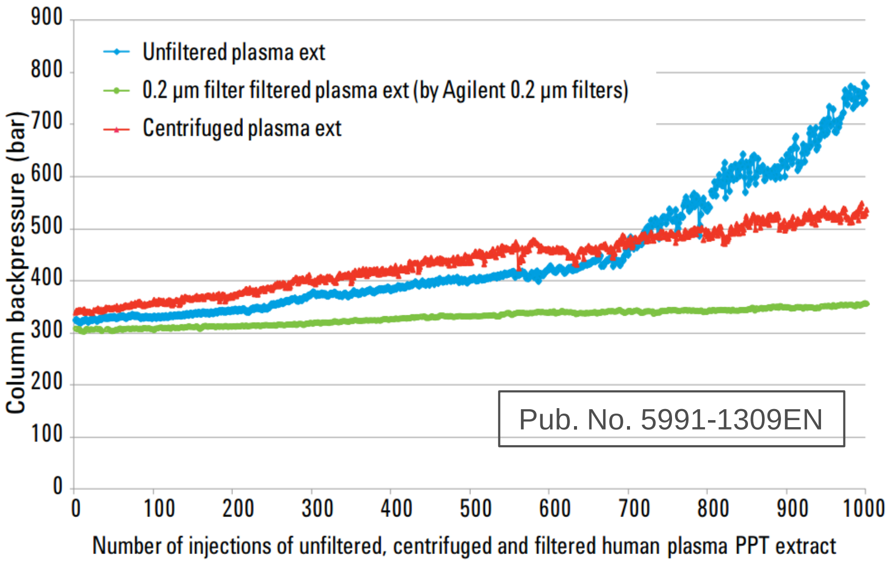
Physically removes particulates from the sample

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

96-well plate formats available



Pub. No. 5991-1309EN

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 EMR-Lipid

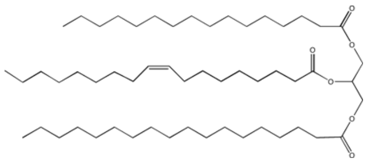
- Captiva EMR-Lipid: 99% lipid removal and protein removal
- Available in 96-well plate, 1 mL, 3 and 6 mL cartridge formats
  - Pass-through/clean-up format
- Solvent retention frit in 96-well plate and 1mL cartridge formats allows for in-well or in-cartridge protein precipitation
- 3 and 6mL cartridge formats do not contain solvent retention frit, which allows for gravity elution
- High analyte recovery
- One step sample addition and activation (20% water is needed to achieve optimum lipid removal)
  - 20% water provided in 3:1-5:1 crash solution for protein precipitation workflow

5991-8006EN	Efficiency of Biological Fluid Matrix Removal using Agilent Captiva EMR-Lipid Cleanup 5991-8006EN
5991-8007EN	Quantitative LC/MS/MS Analysis of Drugs in Human Serum with Agilent Captiva EMR-Lipid Cleanup
5991-7956EN	Vitamin D Metabolite Analysis in Biological Samples Using Agilent Captiva EMR-Lipid 5991-7956EN

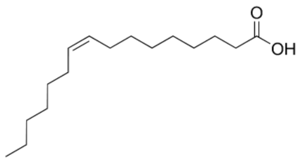
Enhanced Matrix Removal: EMR-Lipid

When "activated" by water…

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

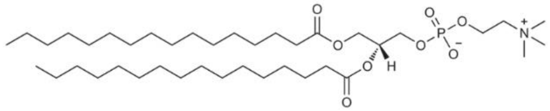


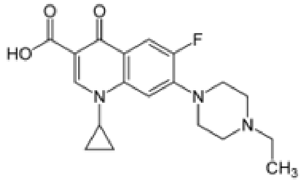
Triglycerides



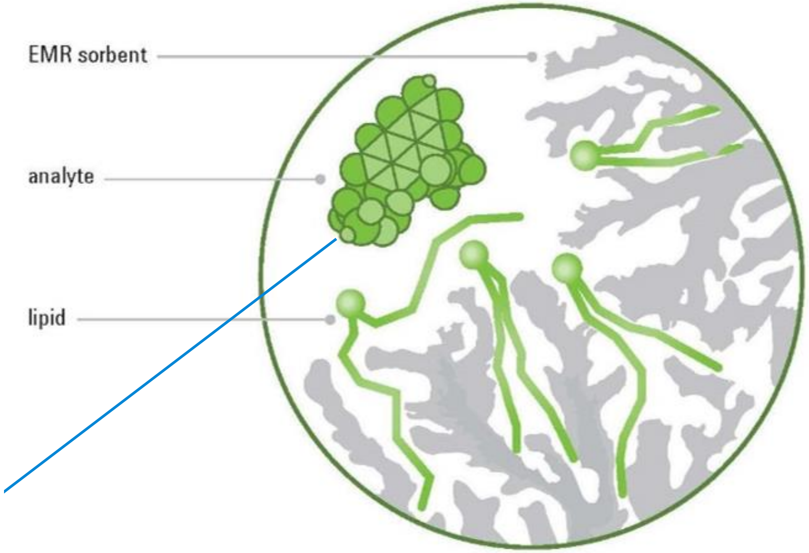
Free Fatty Acids

Phospholipids



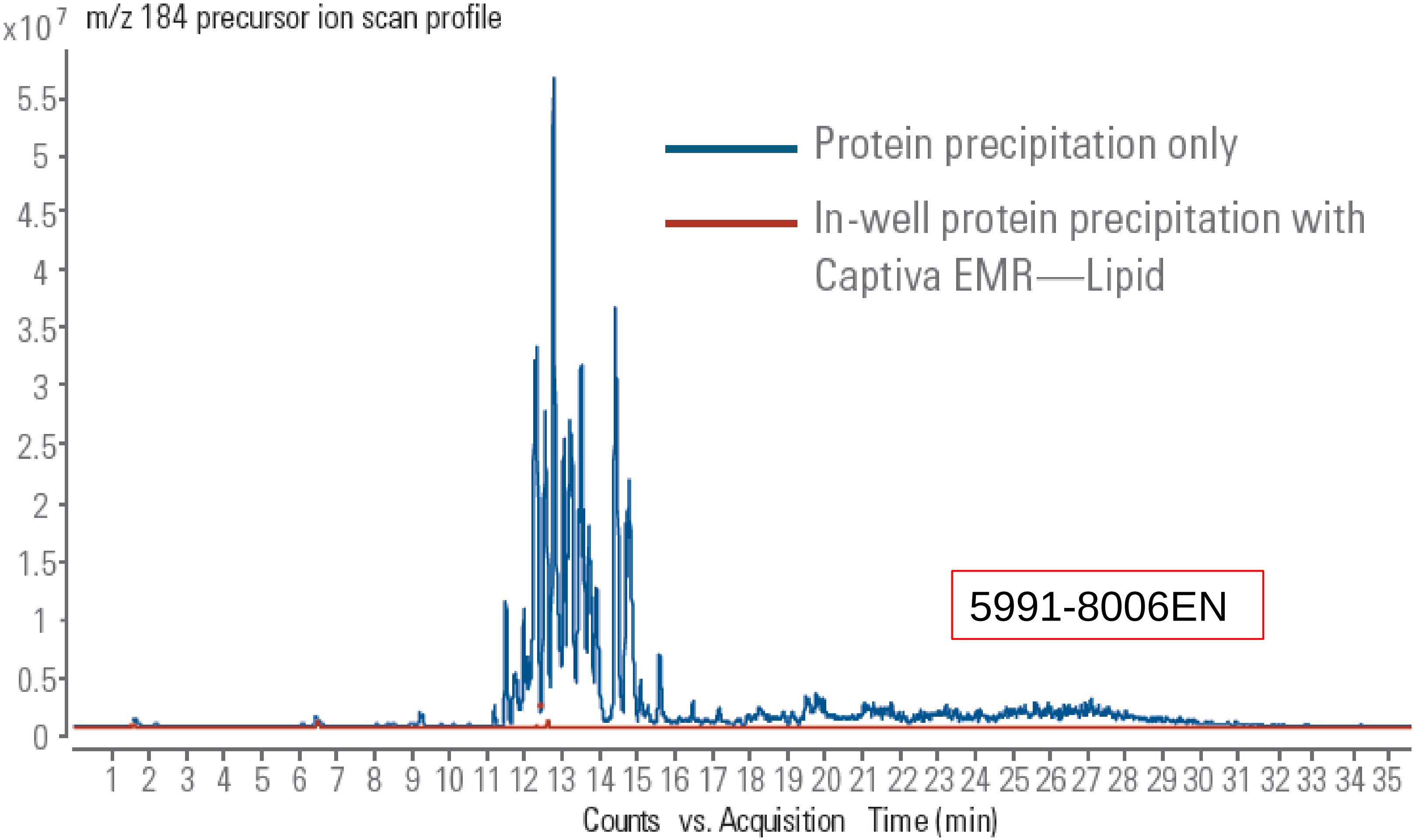


Fluoroquinolones



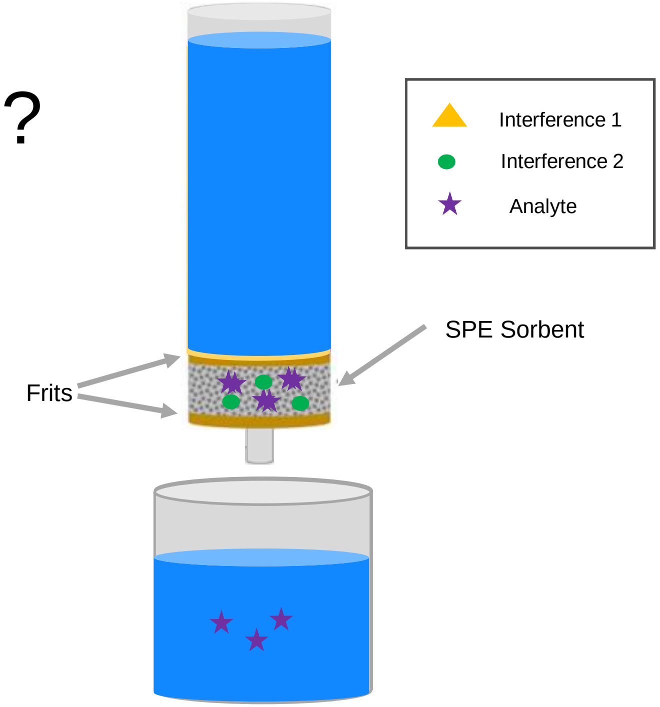
EMR-Lipid Mechanism –
Combined size exclusion and hydrophobic interaction.

Effective phospholipid removal



5991-8006EN

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 hasover 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

Processing 96-Well Plates and Cartridges

Captiva Vacuum Collar

Vac-20 vacuum manifolds

96 well plate vacuum manifold

Positive Pressure Manifolds

COMING SOON

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

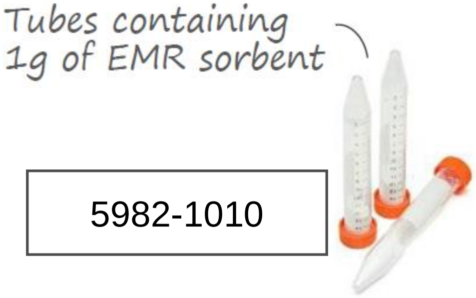
EMR-Lipid: removes unbranched hydrocarbon chains (lipids)

- 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

EMR-Lipid Product Offering

Fits into existing workflows:
- after QUECHERS extraction
- after Liquid Extraction

Effective Removal of lipids (fats)



5982-1010

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

Polish Tube

$NaCl/MgSO_4$

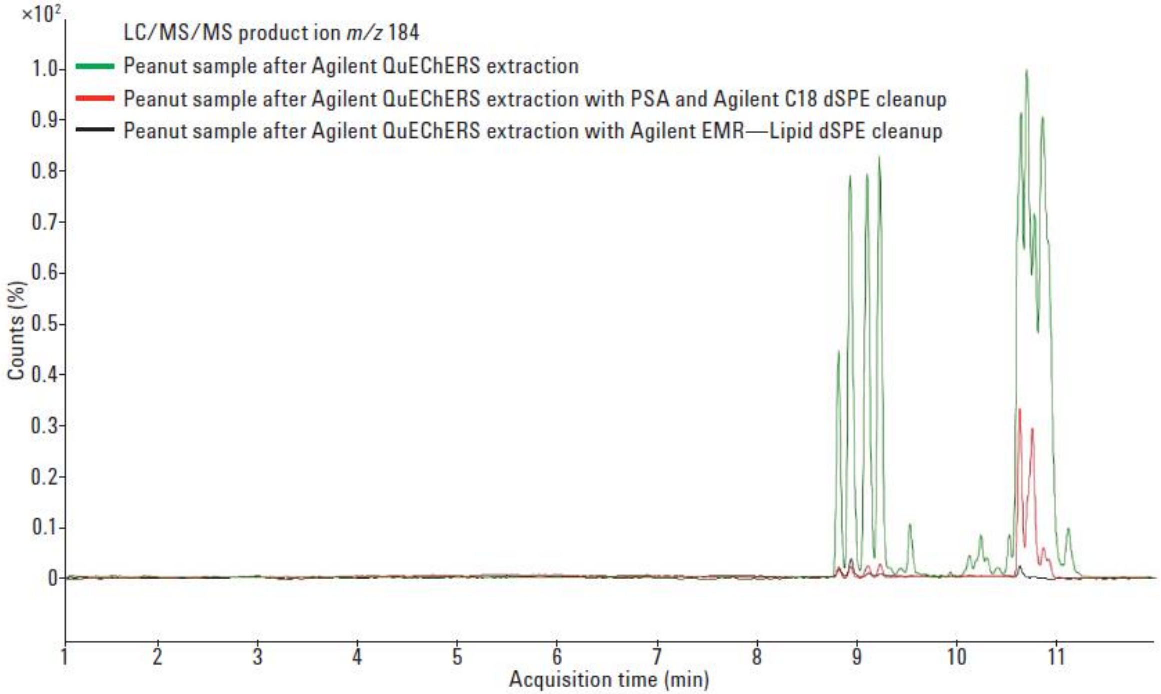
(p/n 5982-0101)

Polish Pouch

$MgSO_4$

(p/n 5982-0102)

Determination of Mycotoxins in Peanuts with Enhanced Matrix Removal-Lipid by LC/MS/MS (5991-7381EN)



A Zorbax Eclipse Plus C18 column was used to separate mycotoxin compounds in peanuts.

A comparison was made for a peanut sample with QUECHERS extraction without cleanup, and with C18/PSA or EMR-Lipid dispersive SPE cleanup.

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 Clean-Up

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

Autosampler Care

Five main maintenance points:

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

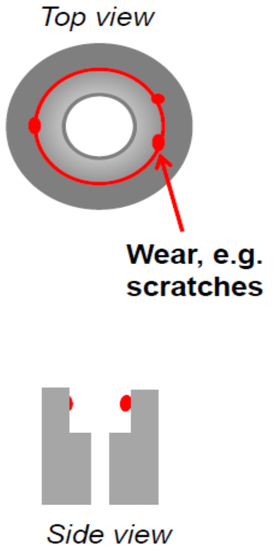
Rotor seal wear - often a cause of sample carry-over

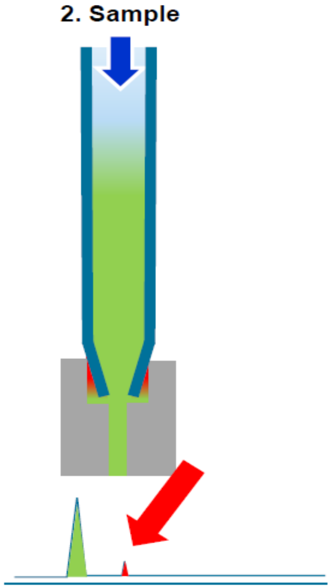
- Exchange the rotor seal
  - After approximately 30000 to 40000 injections, or sooner depending on your specific sample and mobile phase
  - When injection reproducibility or leakage indicates wear

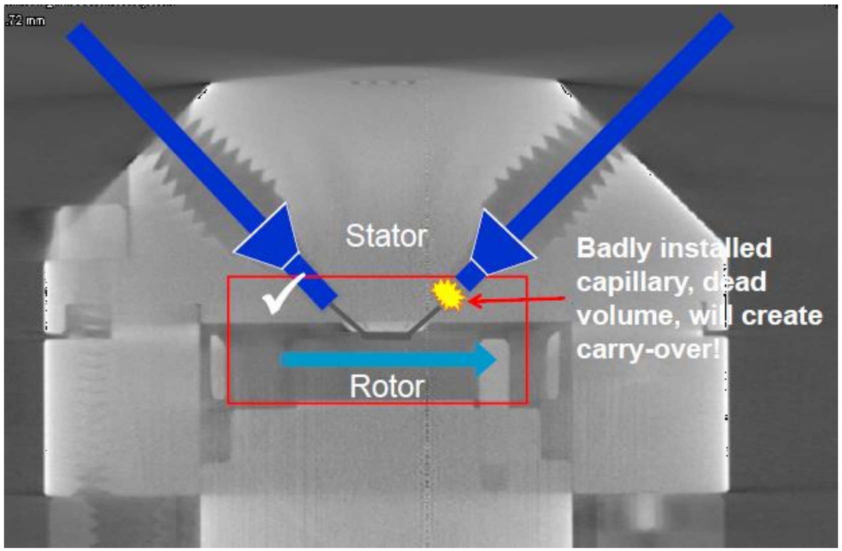
Exchange the metering seal when autosampler reproducibility indicates seal wear

Autosampler Carryover

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







Pressure - Filtration

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 Resists Plugging with 2 µm Frit

Challenging Sample - Plasma

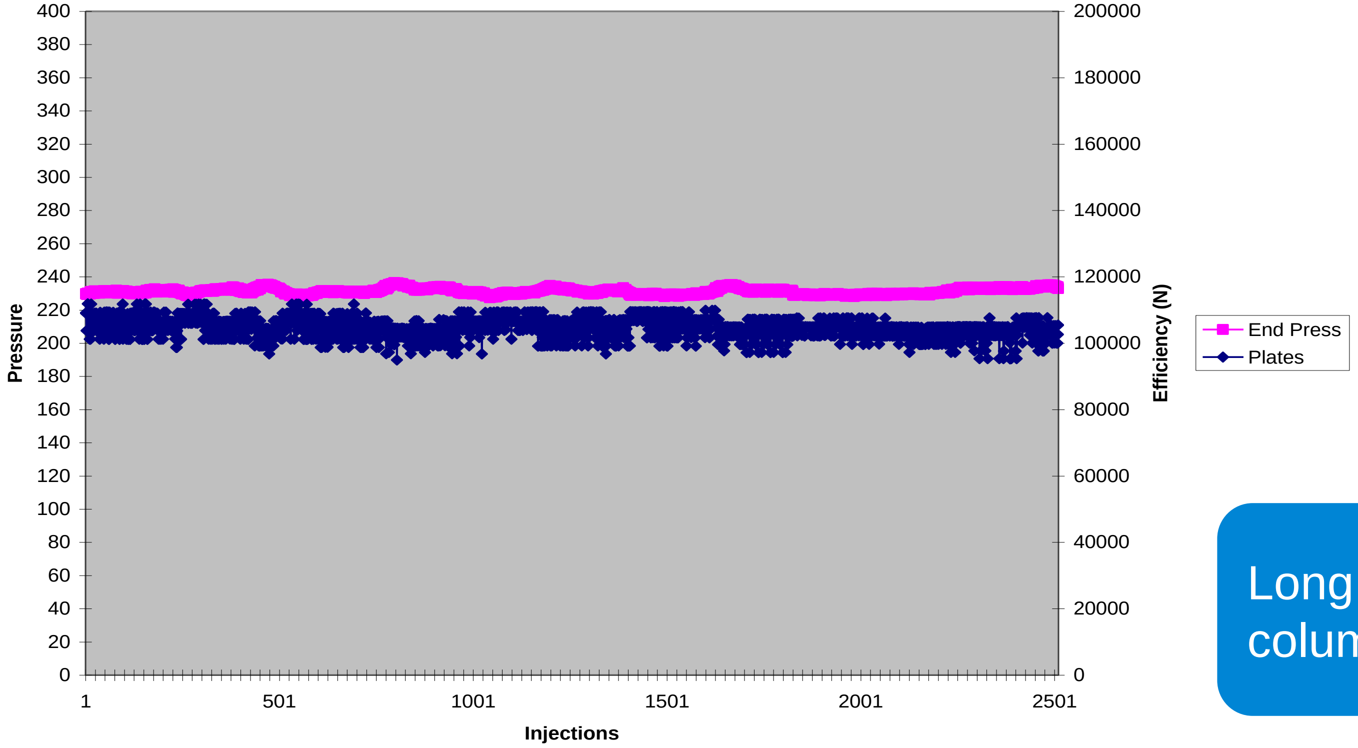
Column: Poroshell 120 EC-C18, 3.0 x 50mm, 2.7um LC: Agilent 1200 RRLC (SL)

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

Injection Volume: 1ul injections

Diflusinal in Plasma



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

Filtration

In-Line Filters

RRLC in-line filter
0.2 µm pore size filter, max 600 bar
- 4.6 mm ID, *5067-1553*
- 2.1 mm ID, *5067-1551*

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

1290 Infinity II LC in-line filter, 0.3 µm -
- 1300 bar, *5067-6189*

- In-line filters can help extend the life of your column
- Not intended to be a replacement for good sample cleanup

Column

Select the Best Column

Low pH

High pH

Stability

Secondary Interactions

Retention

Choose Suitable Column Phase

Mobile Phase pH →

Acidic

SB; Eclipse Plus; EC-C18
pH 1 – 4

SB can use increased temperature 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

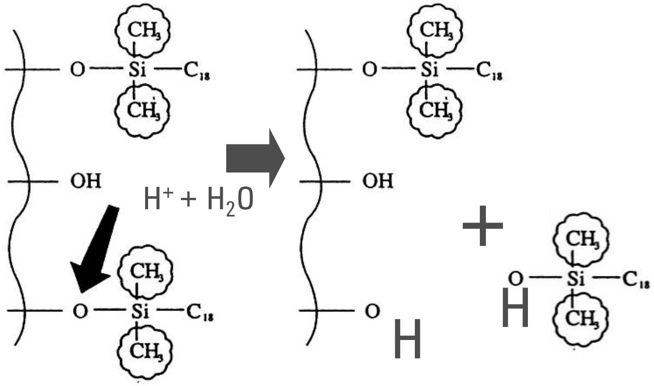
HpH C8 & C18 for high pH

Mechanisms of Degradation of Silica-Based HPLC

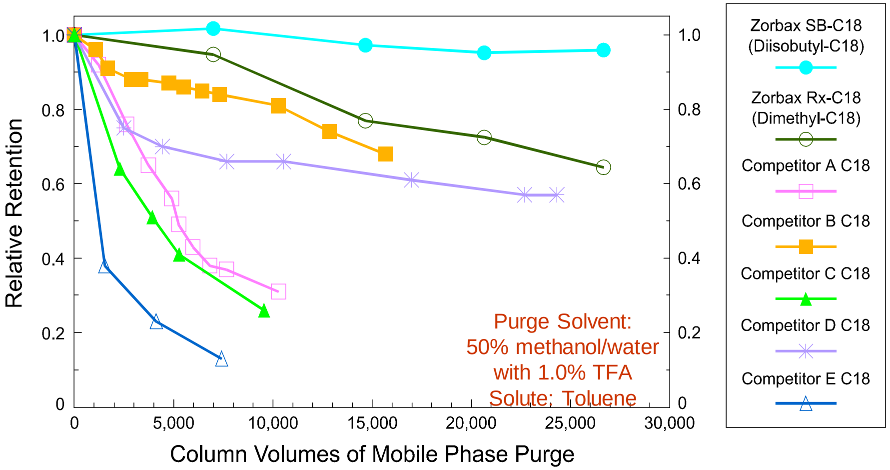
<u>Low pH </u>(pH <3)

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)



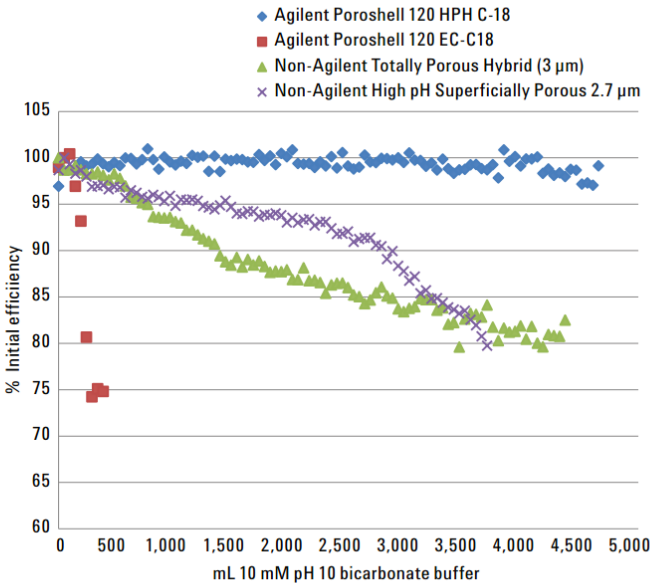
Purge Solvent:
50% methanol/water
with 1.0% TFA
Solute: Toluene

Mechanisms of Degradation of Silica-Based HPLC

Intermediate to High pH(pH >6-7)

Dissolution of the underlying silica by the hydroxide ion

- Loss of silica, void development
- Loss of resolution

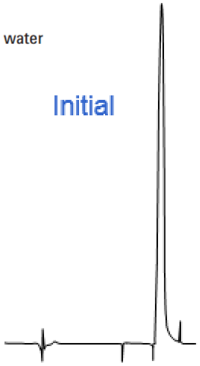


Conditions

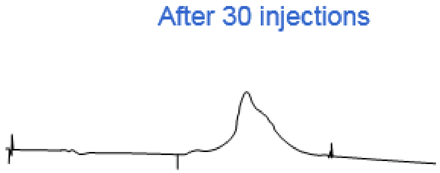
Columns: 2.1 × 50 mm, 2.7 µm
Eluent: A) 0.1% ammonium hydroxide in water
B) acetonitrile
Flow rate: 0.4 mL/min
Gradient:

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

$$SiO^{-}\ Na^{+} + R_3NH^{+} \rightleftharpoons SiO^{-}N^{+}R_3 + Na^{+}$$

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.

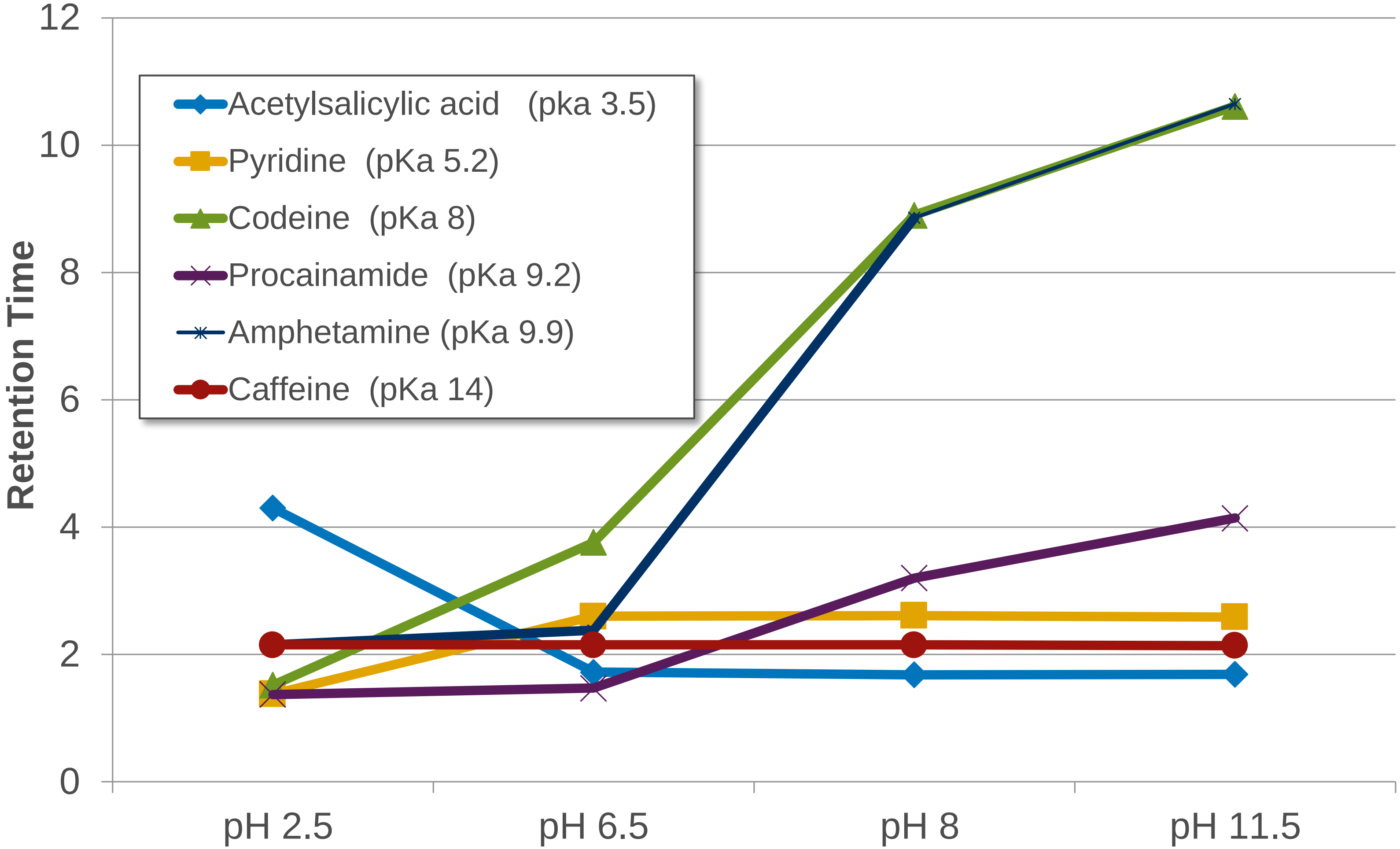
Hydrogen bonding

$$-SiOH + RCOO^{-} \rightleftharpoons -SiO^{-} \ldots H^{+} \ldots {}^{-}OOCR$$

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

Retention Change with pH for Ionizable Analytes Compound-Dependent

Better retention of non-charged analytes (i.e. acids at low pH and bases at high pH)



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

Retention Shifts

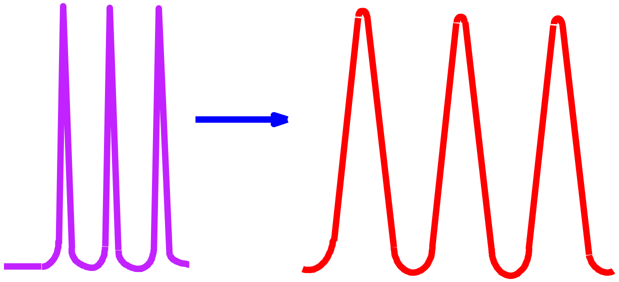
I. All Peaks Shift to Lower Retention (acids, bases, neutrals)
  - Loss of Bonded-Phase
  - Mobile Phase Unstable (less likely)
  - Solvent Delivery System (flow rate)

II. All Peaks Shift to Greater Retention
  - Loss of Organic Solvent in Aqueous / Organic Mix
  - Column Change (less likely)
  - Solvent Delivery System (flow rate)

III. Ionic Peaks Shift Retention
  - Loss of Volatile MP Component (ionic strength, pH shift)
  - Column Change (bonded phase or contamination)

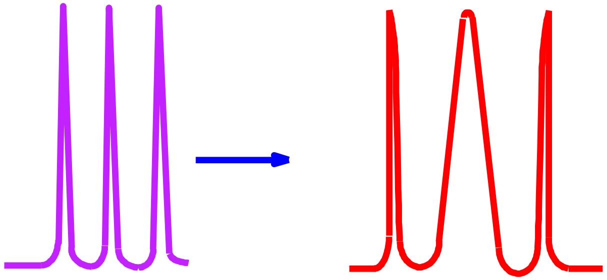
Peak Shape

Peak Shape
Broad Peaks



All Peaks Broadened

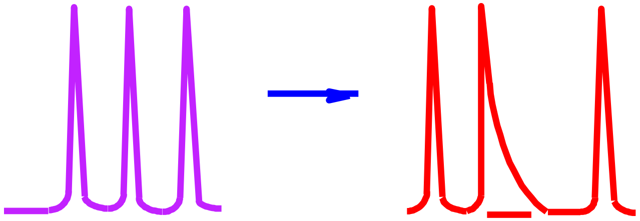
- Loss of Column Efficiency-
  Column Void
- Large Injection Volume / Mass
- High Viscosity Mobile Phase
- Sample Solvent Mismatch

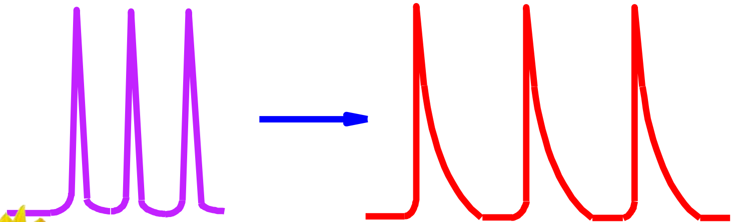


Some Peaks Broadened

- Late Elution from Previous Sample
- High MW Sample - Protein or Polymer

Peak Shape
Tailing Peaks Symmetry >1.5





Causes:

Some Peaks Tail

- Secondary - Retention Effects
  Residual Silanol Interactions
- Small peak Eluting on Tail of Larger Peak

All Peaks Tail

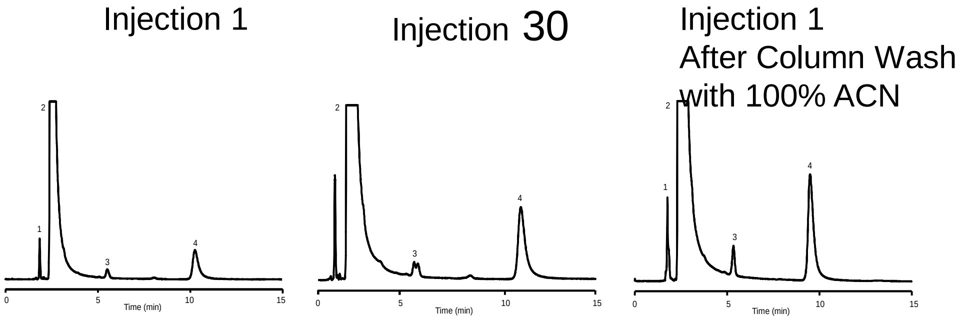
- Extra-Column Effects
- Bad Column
- Build up of Contamination on Column Inlet
- Metals
- Inappropriate Sample Size / Solvent

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



Injection 1

Injection 30

Injection 1
After Column Wash
with 100% ACN

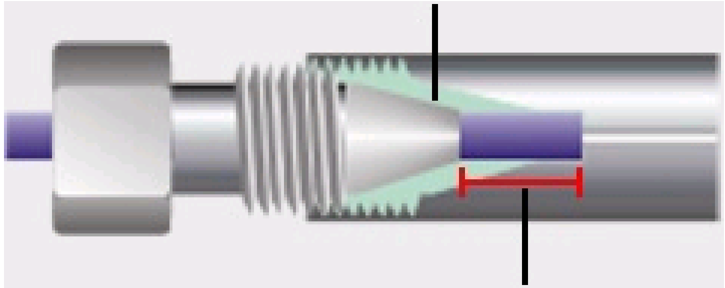
Connections

Quick Connect

Quick Turn

What Happens If the Connections Poorly Made ?

Wrong … too long

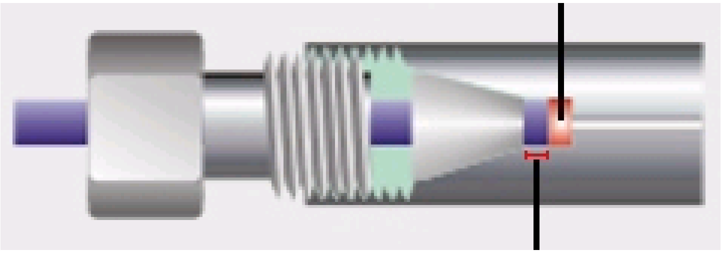


Ferrule cannot seat properly

X

If Dimension X is too long, leaks will occur

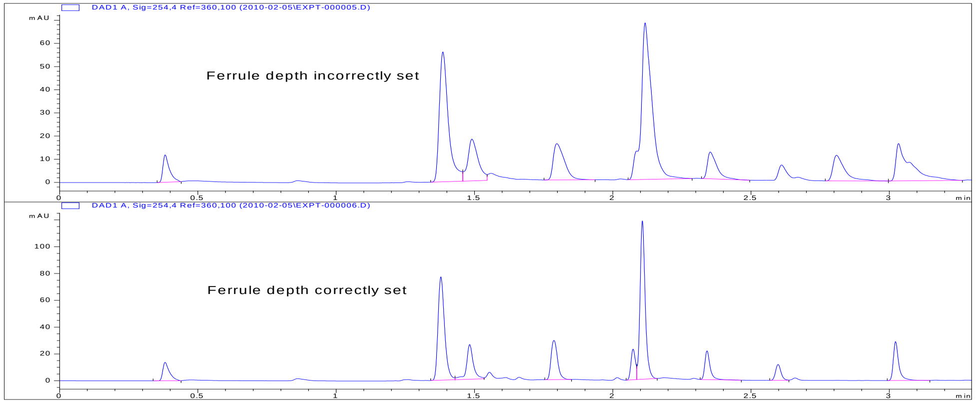
Wrong … too short



Mixing Chamber

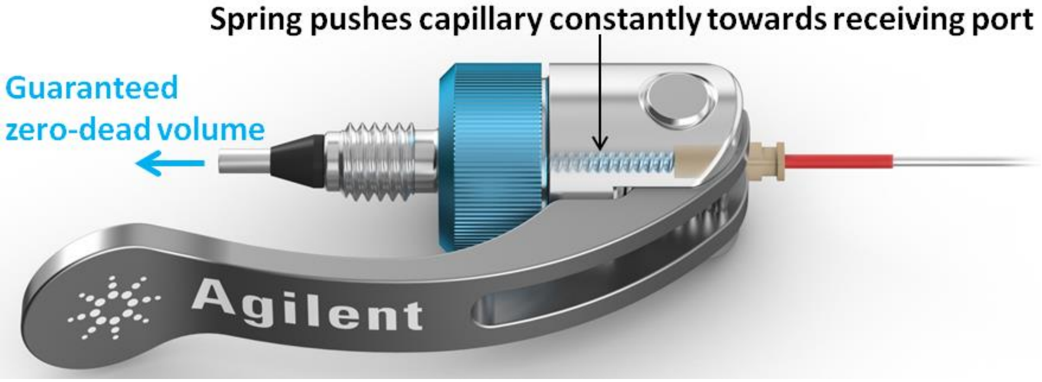
X

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

Simplifying Column & Fitting Connections
A-Line Quick Connect and Quick Turn



Quick Turn fitting

Ease of use functionality

Quick Connect seals with a simple turn of the lever

- Spring-loaded design constantly pushes the tubing against the receiving port
- Delivers reproducible connection with no dead volume
- consistent chromatographic performance
- **Quick Connect Finger-tight to 1300bar**

Quick Turn

- Stem length adjustable through spring
- makes the fitting compatible with all types of LC columns
- **Finger-tight to 600bar, wrench-tight to1300bar**

Detector Care
UV Detectors

DAD tungsten lamp

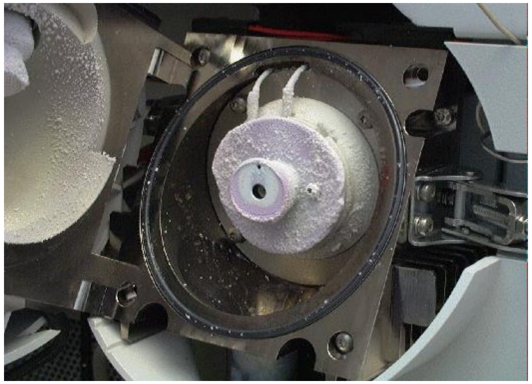
Two types

- VWD
- DAD/MWD

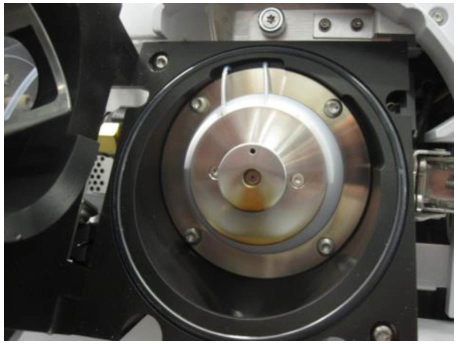
Simple Maintenance

- ✓ Lamp replacement
- ✓ Flow cell cleaning or replacement
- ✓ Know the pressure rating of your flow cell – another detector or 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 flow cell contains 5-10% organic when not in use - prevents microbial growth
- ✓ Avoid leaving buffer solutions in the flow cell - can crystallize

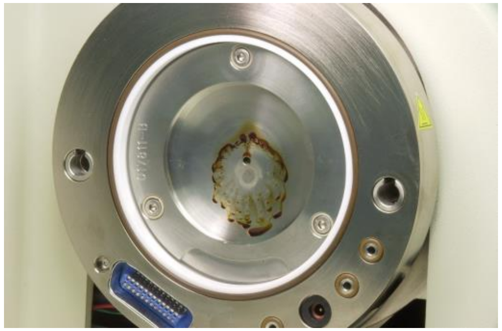
Examples of Instrument Contamination/Road Blocks



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)

- Maintenance videos https://www.youtube.com/playlist?list=PLThrdl2ragolmT3J-W5r8ailvJN94DJMR

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

Choose the best column for your sample and conditions

Contact Agilent Chemistries and Supplies Technical Support

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

Option 1 for GC/GCMS Columns and Supplies

Option 2 for LC/LCMS Columns and Supplies

Option 3 for Sample Preparation, Filtration and QuEChERS

Option 4 for Spectroscopy Supplies

Available in the USA & Canada 8-5 all time zones

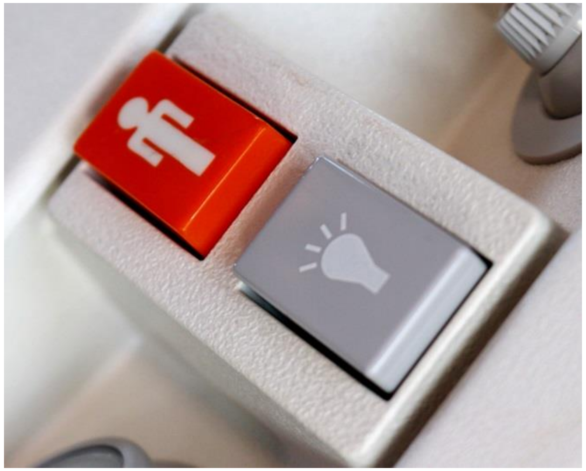
gc-column-support@Agilent.com

lc-column-support@agilent.com

spp-support@agilent.com

spectro-supplies-support@agilent.com

Additional Resources and Application Support



Sample preparation eSeminar Series

https://www.agilent.com/en-us/training-events/eseminars/sample-preparation

Reference Materials and Guides:

Agilent Enhanced Matrix Removal – Lipid Brochure (Publication Number: 5991-6052EN)

https://www.agilent.com/cs/library/brochures/EMR%20Brochure%20CPOD%20Final_LoResSglPgs.pdf

https://www.agilent.com/en-us/products/sample-preparation/sample-preparation-methods/sample-preparation-methods/enhanced-matrix-removal-lipid

Agilent Sample Preparation Landing Page

https://www.agilent.com/en-us/products/sample-preparation/sample-preparation-methods

Agilent Sample Preparation Catalog (Publication Number: 5991-1057EN)

http://www.agilent.com/cs/library/catalogs/public/5991-1057EN%20Sample%20Prep%20Catalog.pdf

Agilent Sample Preparation Products Technical Support Contact Information*:

Phone: 800-227-9770, Options 3, 3, 3

Email: spp-support@agilent.com

* *North America*

Appendix

Typical Schedule*

PUMPS

Item	Typical Schedule	Comments
Solvent inlet filter	Replace every 6 - 12 months	
PTFE frits in purge valve + gold seal	Every 12 months	
Piston seals	Every 12 months	When changing the seal, check the piston for scratches--replace if scratched
Inlet valve cartridge, outlet ball valve	Every 24 months	

AUTOSAMPLER

Item	Typical Schedule	Comments
Needle and needle seat	Every 12 months	
Rotor seal	Every 12 months	
Metering device seal	Every 24 months	

COLUMN COMPARTMENT

Item	Typical Schedule	Comments
Column switching valve rotor seal	Every 12 months	
Column fittings	Every 5 to 10 column changes	A-line fittings last a lot longer than traditional fittings

DETECTORS

Item	Typical Schedule	Comments
Lamps	Every 2000 hours	Watch for a noisy baseline
Flow cell	Check cleanliness every 6 months	Low light intensity could be caused by a dirty flow cell

*Adjust according to your samples, conditions, and performance goals

Poroshell 120 Column Chemistries

Multiple bonded phases for flexibility in method development

2.7µm, 4 µm, 1.9 µm

12 chemistries

Poroshell 120 EC-C18 and C8

- Robust end-capped C18/C8 for best peak shape at pH 2-9

Poroshell 120 Stablebond C18 and C8

- Robust chemistries for pH<2
- Non-endcapped for alternate selectivity

Poroshell HPH-C18 and HPH-C8

- Long lifetime at high pH

Poroshell 120 Phenyl-Hexyl

- Excellent choice for pi-pi interactions
- Selectivity similar to phenyl, diphenyl, or other phenyl-hexyl columns

Poroshell 120 SB-Aq

- Proprietary bonded phase is an excellent choice for polar analytes

Poroshell 120 Bonus-RP

- Embedded polar group provides unique selectivity for polar compounds

Poroshell 120 EC-CN

- Flexible end-capped CN chemistry for normal and reversed-phase separations

Poroshell 120 HILIC

- Bare silica HILIC for use in hydrophilic interaction chromatography of polar molecules

Poroshell 120 PFP

- Pentafluorophenyl chemistry for orthogonal selectivity relative to C18

Poroshell 120 Column Chemistries

Bonded Phase	Pore Size (Å)	Temp. Limit (°C)	pH Range	Endcapped	Carbon Load (%)	Surface Area (m²/g)
EC-C18	120	60	2 - 8	Double	10	130
EC-C8	120	60	2 - 8	Double	5	130
SB-C18	120	90	1 - 8	No	8	130
SB-C8	120	80	1 - 8	No	5.5	130
HPH-C18	100	60	3 - 11	Double	Proprietary	95
HPH-C8	100	60	3 - 11	Double	Proprietary	95
Phenyl-Hexyl	120	60	2 - 8	Double	9	130
SB-Aq	120	80	1 - 8	No	Proprietary	130
Bonus-RP	120	60	2 - 9	Triple	9.5	130
HILIC	120	60	0 - 8	No	N/A	130
EC-CN	120	60	2 - 8	Double	3.5	130
PFP	120	60	2 - 8	Yes	5.1	130