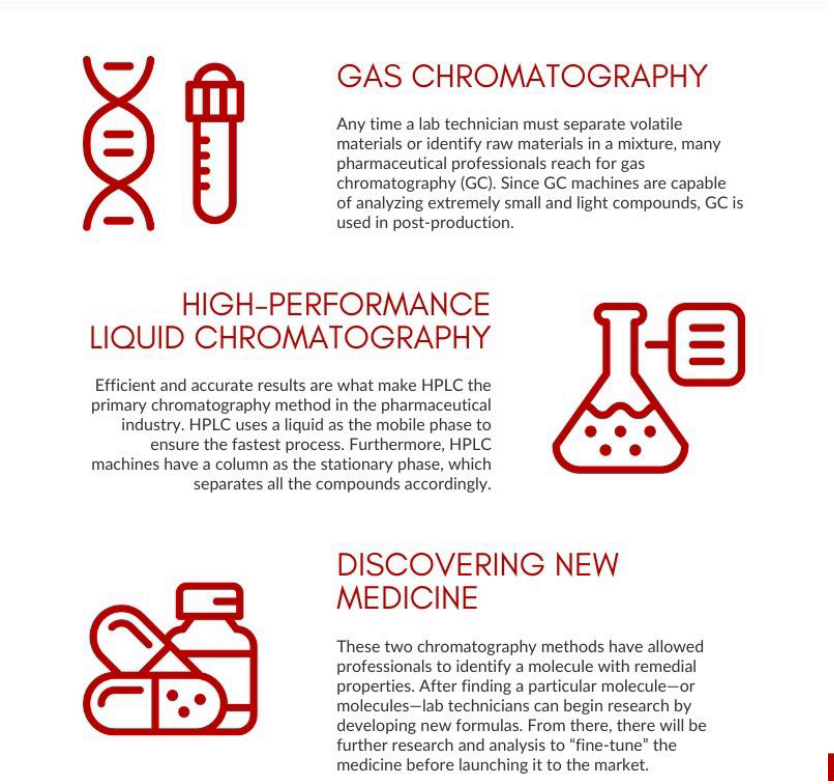
Mobile Phase Preparation, Storage, & Handling

- Developer of Russia's Sputnik V Covid-19 vaccine claims there were no cases of blood clots encountered during trials & use of vaccine
  - …pointed to a recent study, published in The New England Journal of Medicine, that linked blood clots in those inoculated to insufficient purification of vaccines.
  - *Unlike other vaccines,"* the Russian [dose] goes through a four-stage purification process, **consisting of two stages of chromatography** and two stages of tangential flow filtration, which assures its *"quality and safety,"* the statement read.

https://sputnikvaccine.com/about-vaccine/ & https://www.rt.com/russia/521047-sputnik-blood-clots-astrazeneca/

** Note this is state run media

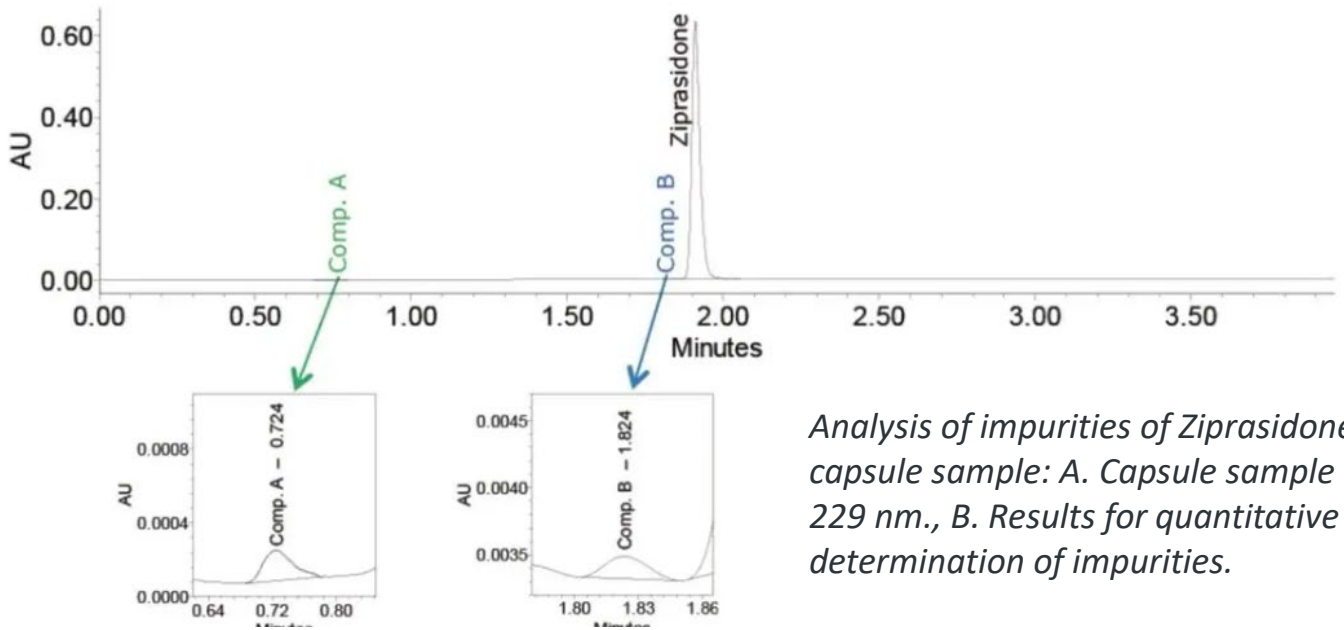
Chromatography in Pharma



https://gentechscientific.com/chromatography-use-in-pharmaceutical-industry/

Impurity Analysis of Ziprasidone

A.



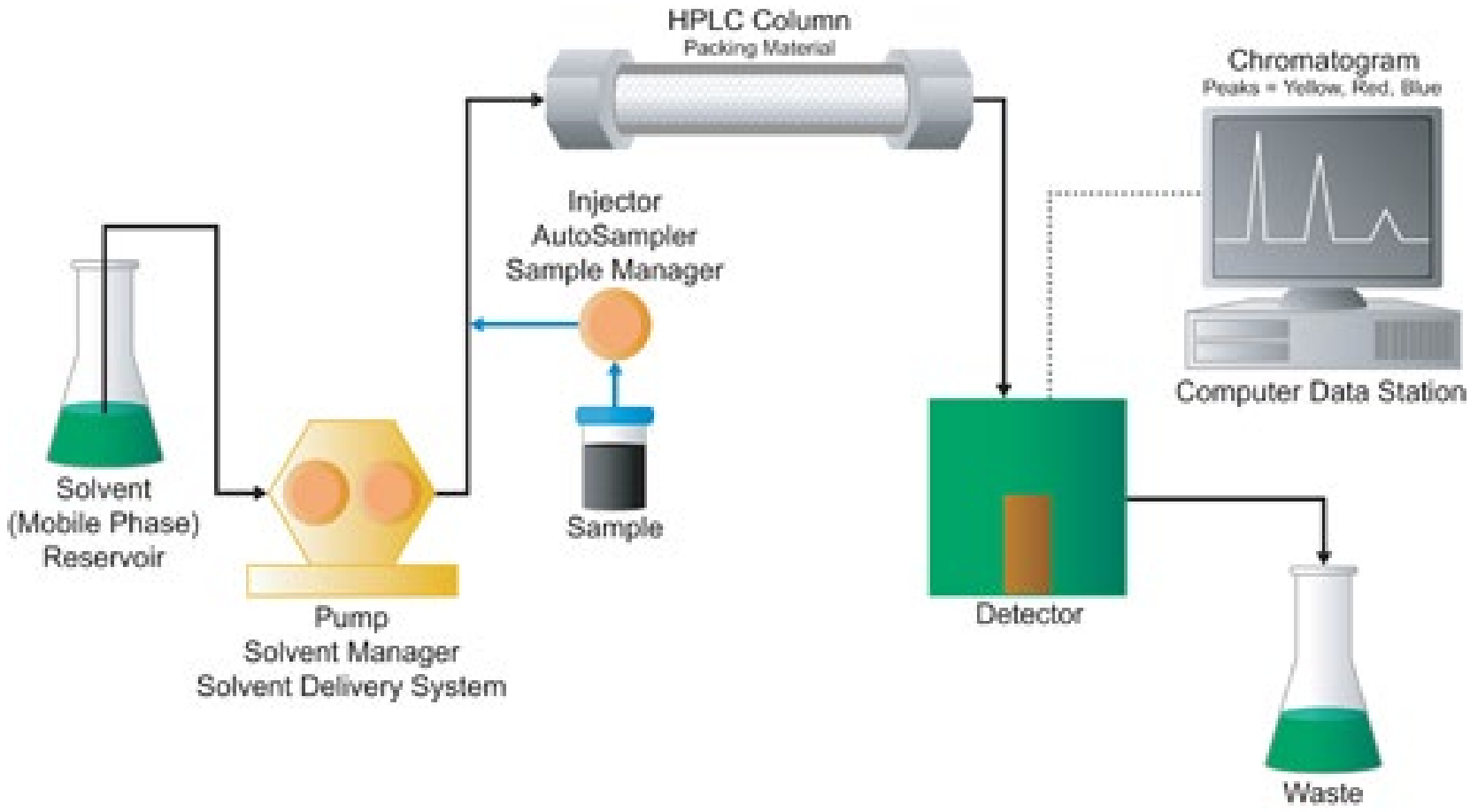
Analysis of impurities of Ziprasidone HCl in the capsule sample: A. Capsule sample with UV at 229 nm., B. Results for quantitative determination of impurities.

B.

	Name	Component Type	Retention Time (min)	Area (µV*sec)	Impurity Response	Above Reporting Threshold	Above Identification Threshold	Above Qualification Threshold	ICH Threshold	Pass?
1	*Compound A*	Specified Impurity	0.724	439	*0.027*	☑	☑	☑	Above Qualification Threshold	Fail
2	Compound B	Specified Impurity	1.824	230	0.017	☐	☐	☐	Below Reporting Threshold	Pass
3	Ziprasidone	Main Component	1.911	1145524						
4	Compound D	Low Level Impurity	2.978							
5	Compound C	Low Level Impurity	3.119							
6	Sum Specified Impurities	Specified Impurity		669	0.044					Fail

https://www.waters.com/nextgen/ca/en/library/application-notes/2014/analysis-impurities-pharmaceutical-products-empower-ich-impurity-processing.html

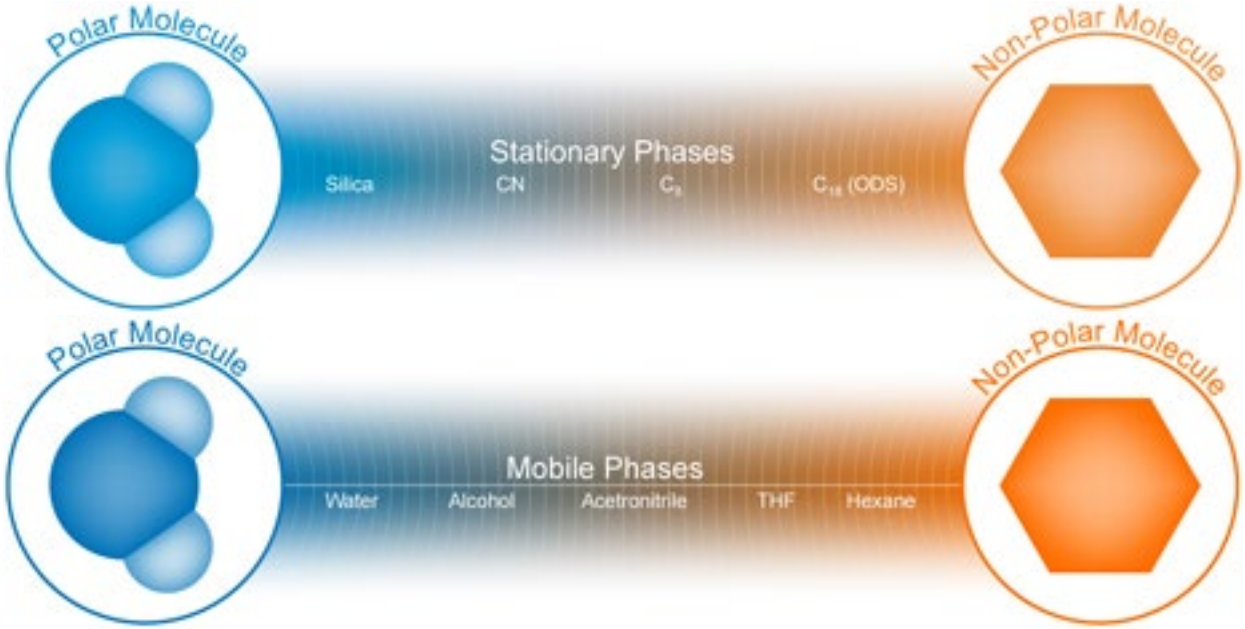
High Performance Liquid Chromatography



Courtesy of Waters Canada

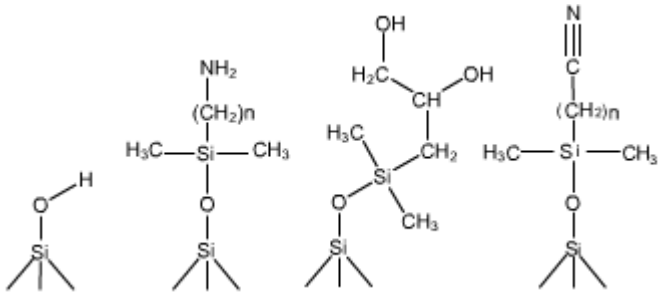
Creating Separations

- Typically want to have analyte retained somewhat by column
  - Choose a SP with a similar polarity
  - Choose a MP with a dissimilar polarity



Normal Phase Chromatography

- SP = polar (more than MP)
  - Ex. Silica, cyano, diol amino
- MP = non-polar (less polar than SP)
  - Ex. Hexane

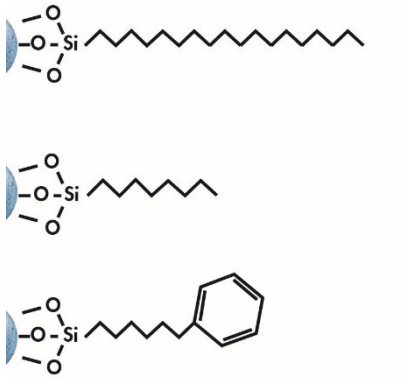


- Polar compounds retain most strongly
- Non-polar elute more easily
- Called “normal” bc it was the first type of partition separation developed

Image: Waters Canada

Reverse Phase Chromatography

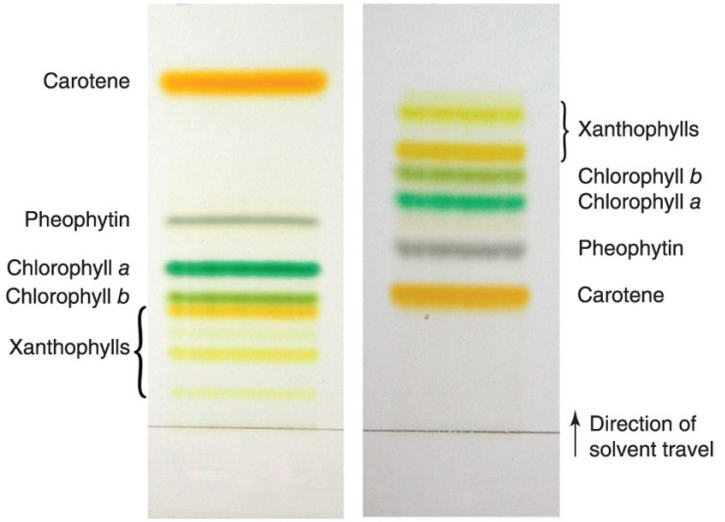
- Most common form of liquid chromatography
  - HPLC typically refers to RP
- SP = non-polar (more than MP)
  - Ex. C18, C8, C4, phenyl
- MP = polar (more than SP)
  - Ex. Water, MeOH, ACN



- Non-polar compounds retain most strongly
- Polar elute more easily
- Called “reverse” bc it is the opposite of normal phase

Demonstration 25-1 Normal- and Reversed-Phase Chromatography (3 of 3)

- The normal-phase separation uses a 1 × 6.5-cm silica thin-layer chromatography plate and is developed in a 1.5 × 10-cm test tube using ≤1 mL of 70:28:2 (vol:vol:vol) heptane:acetone:anhydrous ethanol.
- The reversed-phase separation uses a 1 × 6.5-cm C_{18} thin-layer chromatography plate and is developed in a 1.5 × 10-cm test tube using ≤1 mL of 15:35:50 (vol:vol:vol) heptane:acetonitrile:95% ethanol.
- With a pencil, gently draw a line ~0.7 cm from the end of the plate.
- Use a capillary tube to apply a series of spots along the horizontal line.
- Add eluent to the test tube and place the plate in the test tube (make sure the eluent does not rise above the horizontal line).
- Remove the plate when the solvent front is 0.5–1.0 cm from the top (<10 min).



(*a*) Normal-phase (*b*) Reversed-phase

Courtesy of L. Kvittingen, Norwegian University of Science and Technology. From B. J. Sjursnes, L. Kvittingen, and R. Schmid, "Normal and Reversed-Phase Thin Layer Chromatography of Green Leaf Extracts," *J. Chem. Ed.* **2015**, *92*, 193. Reprinted with permission © 2015, American Chemical Society.

Simplified RP Retention Mechanism

- Retention based on hydrophobic interactions
- The high polarity of the water “repels” the non-polar analytes out of the MP and onto the SP
  - The concentration of the analyte on the SP increases

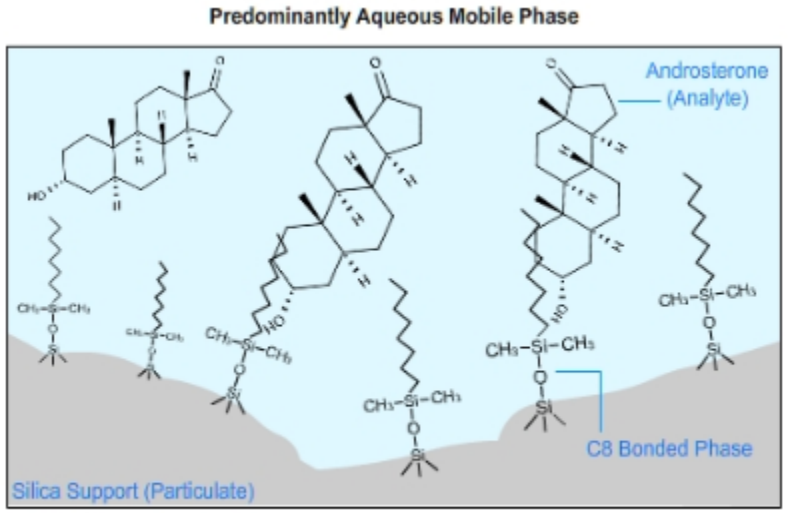
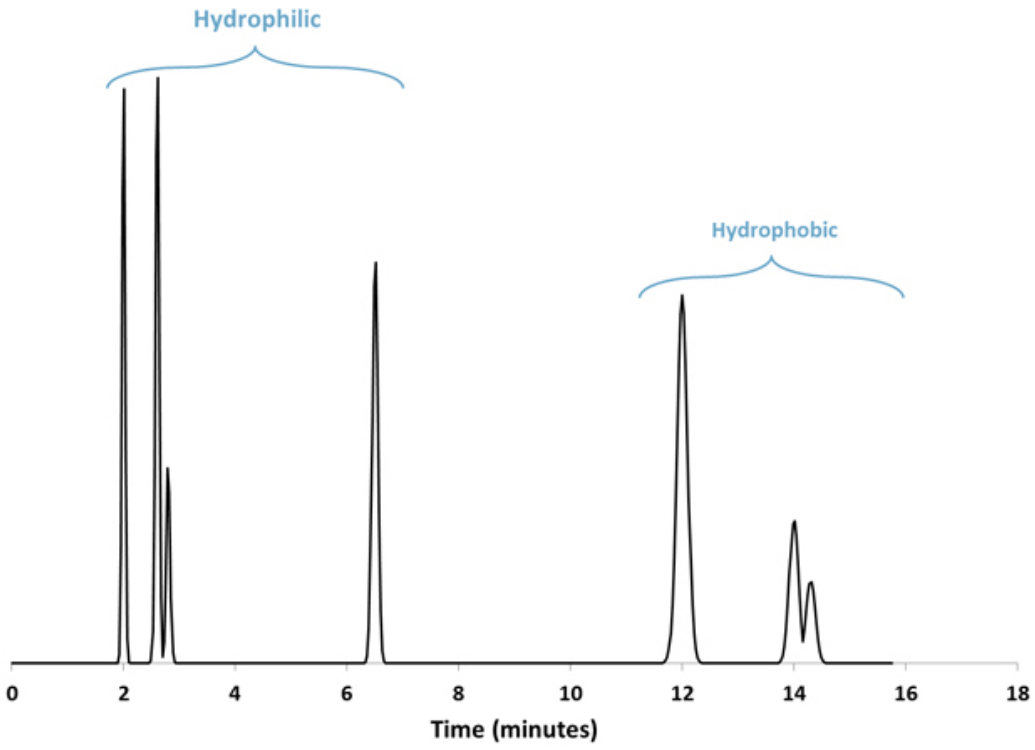


Figure 1: Schematic representation of Reversed Phase HPLC

Determination of Elution Order

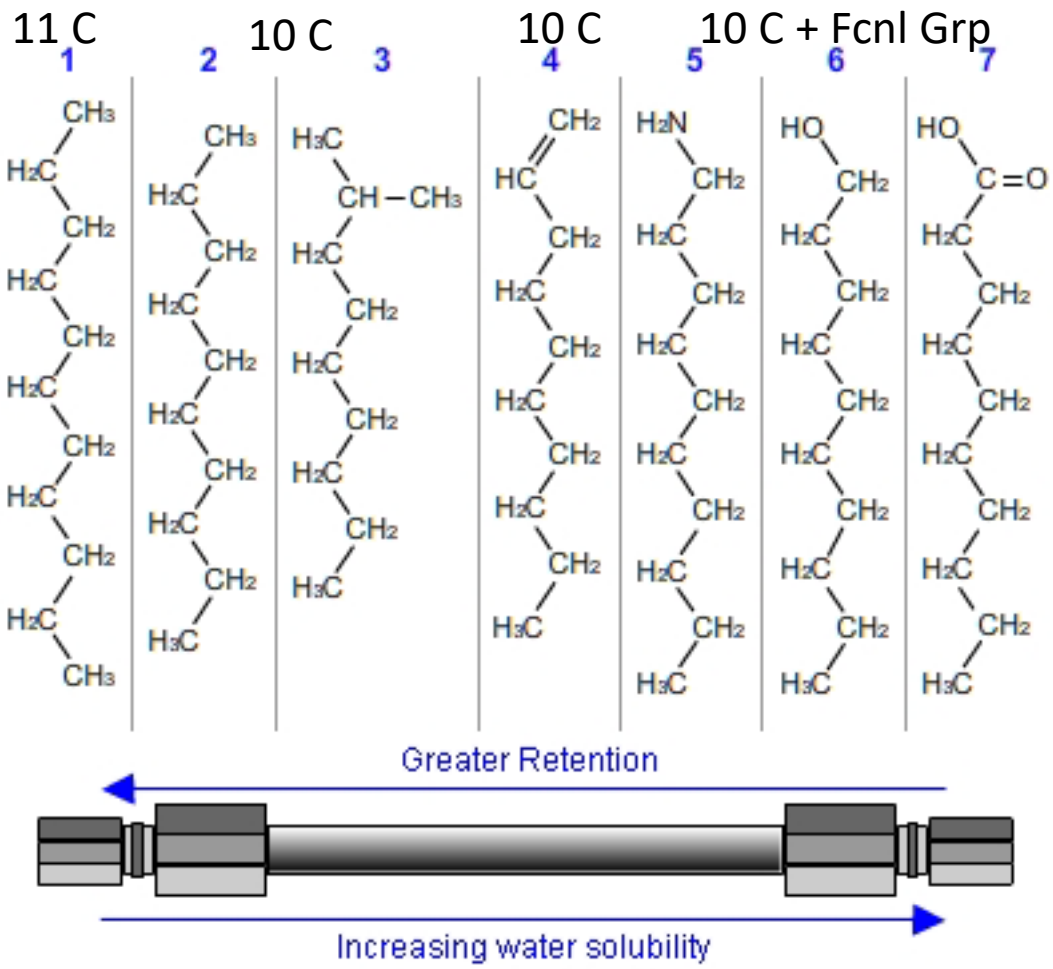
- Hydrophilic/polar compounds will elute first



Elution Order

- The structure of a sample gives clues as to elution order
- When comparing SIMILAR compounds
  - Less water soluble = more retention
  - More carbon atoms = more retention
  - Branched chain compounds = less retention
  - Unsaturation = less retention
  - Polar and charged species = less retention
- These are GENERALIZATIONS
  - SP and MP dependent

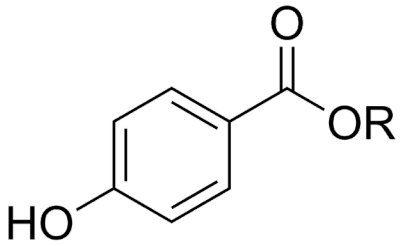
General Trends in Elution

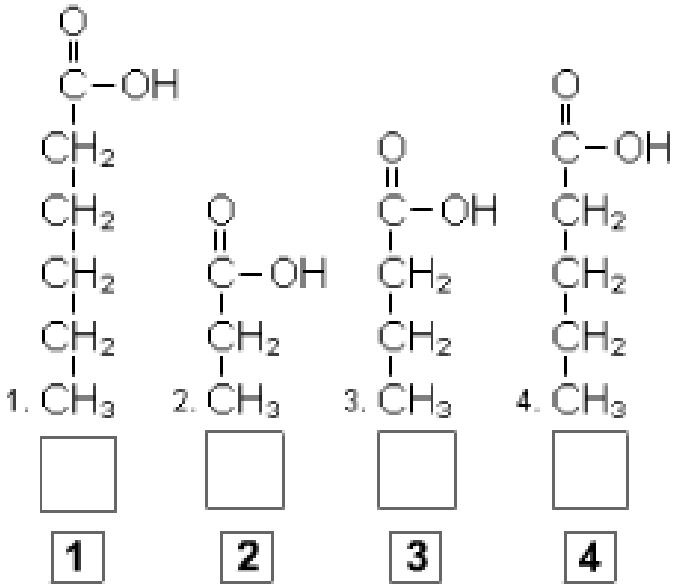


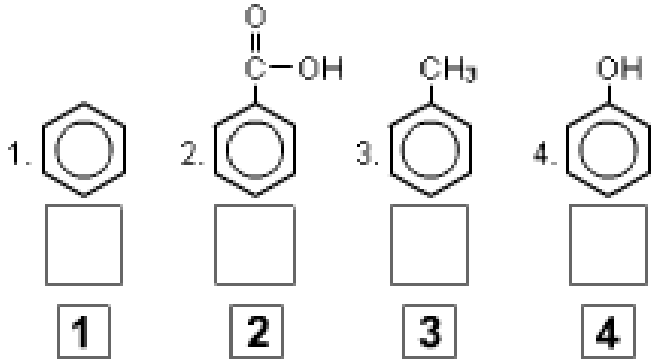
Note this is a homologous series!

Practice

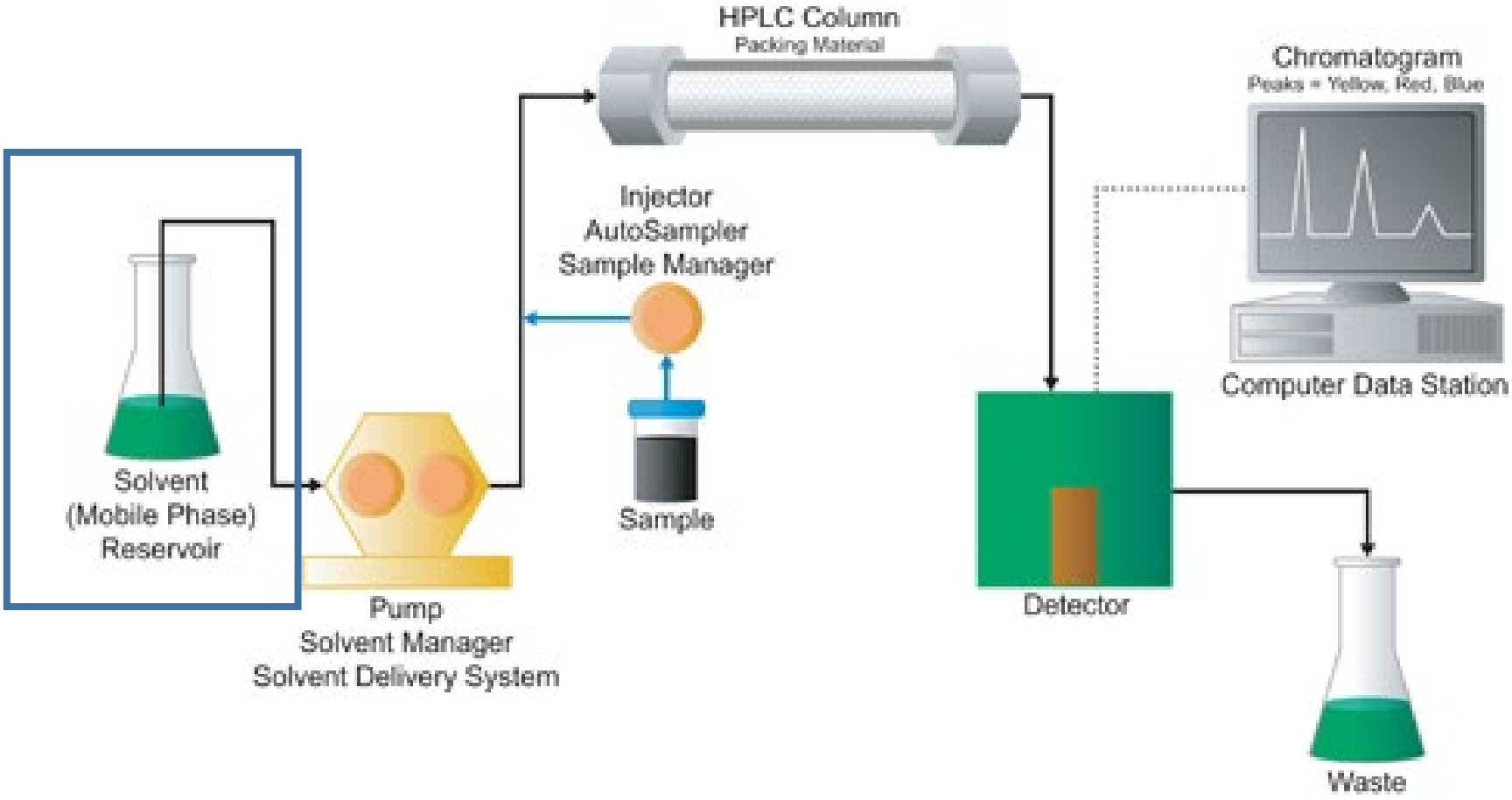
- Indicate the elution order by RP (1=first)
- Butyl, ethyl, methyl, propyl paraben







High Performance Liquid Chromatography

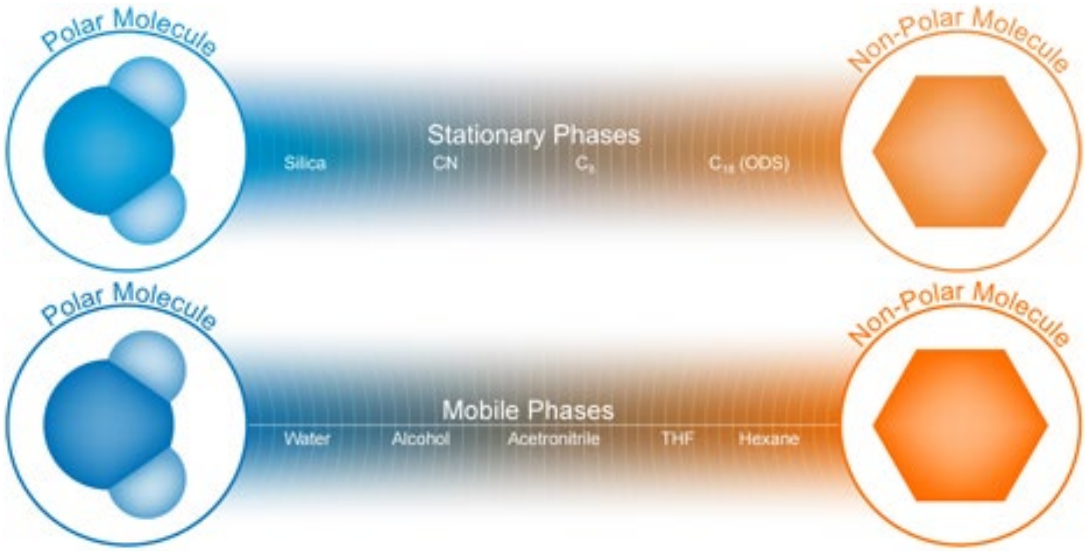


Solvent Strength

- When we make the mobile phase more similar to the stationary phase we increase its strength
  - NP – make it more polar
  - RP – make it more non-polar
- The stronger the solvent, the faster compounds elute
  - Regardless of analyte properly

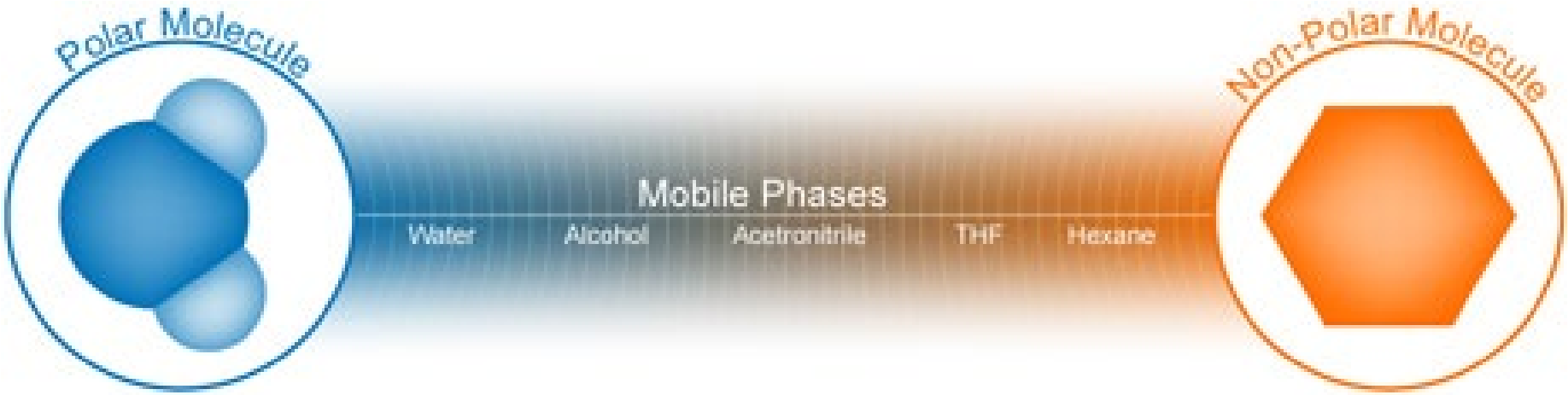
Mobile Phases in RP

- Water is least like the SP
  - Typically termed the “weak solvent”
- MP that are more like the SP are “stronger”
  - Elution faster

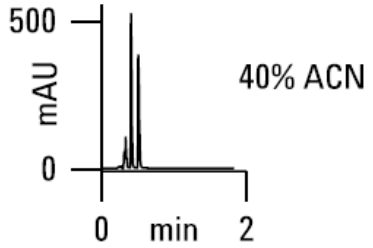


Strength of the Mobile Phase

- We can change the “strength” of the MP in two ways for RP separations
  1. Increasing the % organic in our mixture
     - 30:70 MeOH:water vs 70:30 MeOH:Water
  2. Changing to a less polar solvent
     - Water to MeOH or ACN or THF

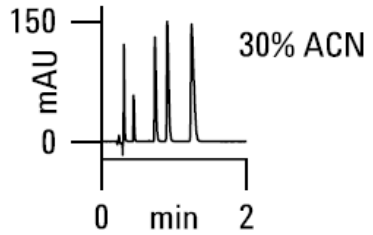


RP Isocratic – Increasing % Organic

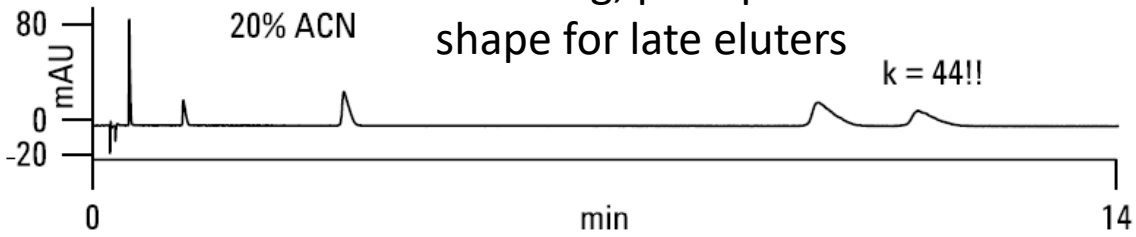


Not all of the peaks are resolved

Peak identification
1. Pindolol
2. Diisopyridamide
3. Propranolol
4. Dipyridamole
5. Diltiazem



- Good resolution
- Fast analysis
- No time wasted

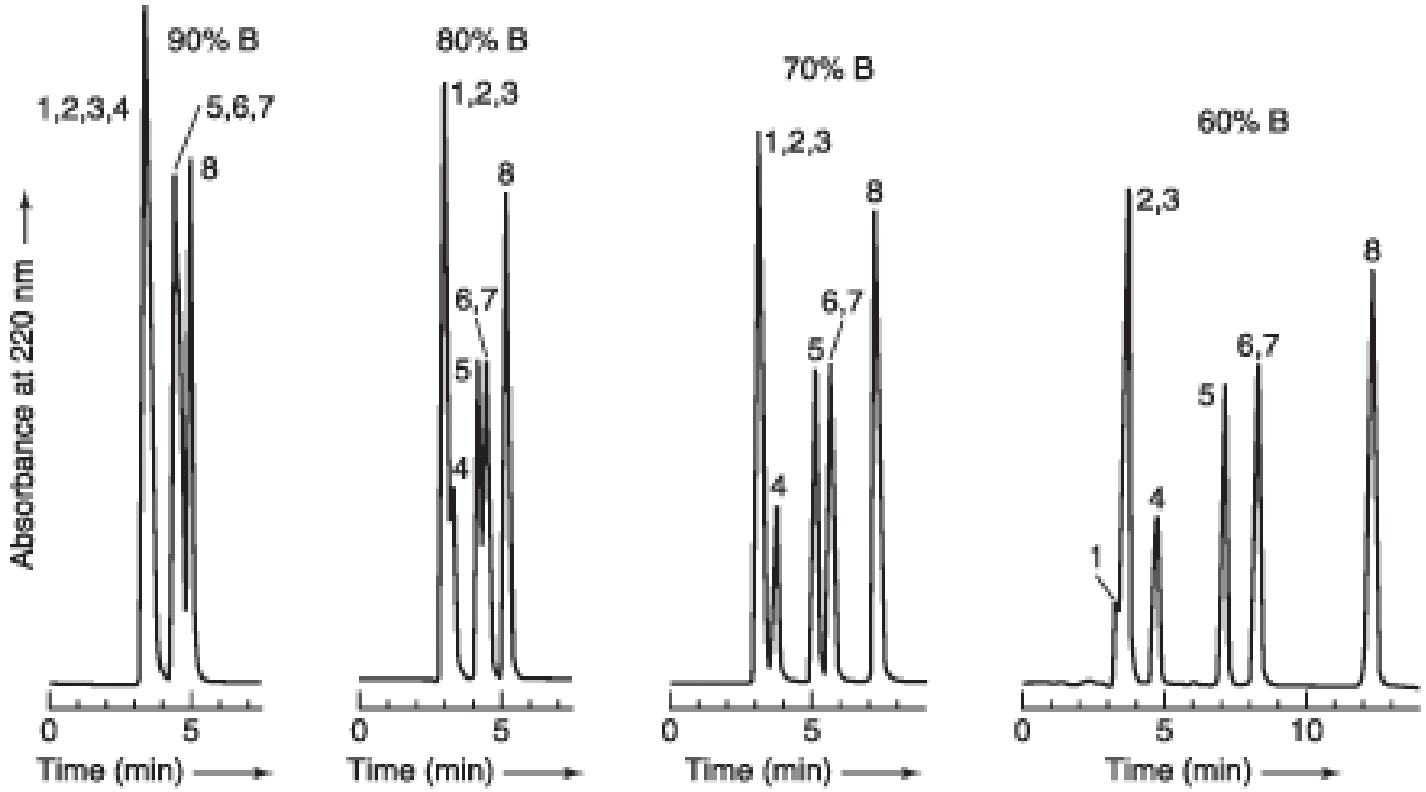


Too long, poor peak shape for late eluters

Increasing % organic reduces retention time
- Organic is the strong solvent

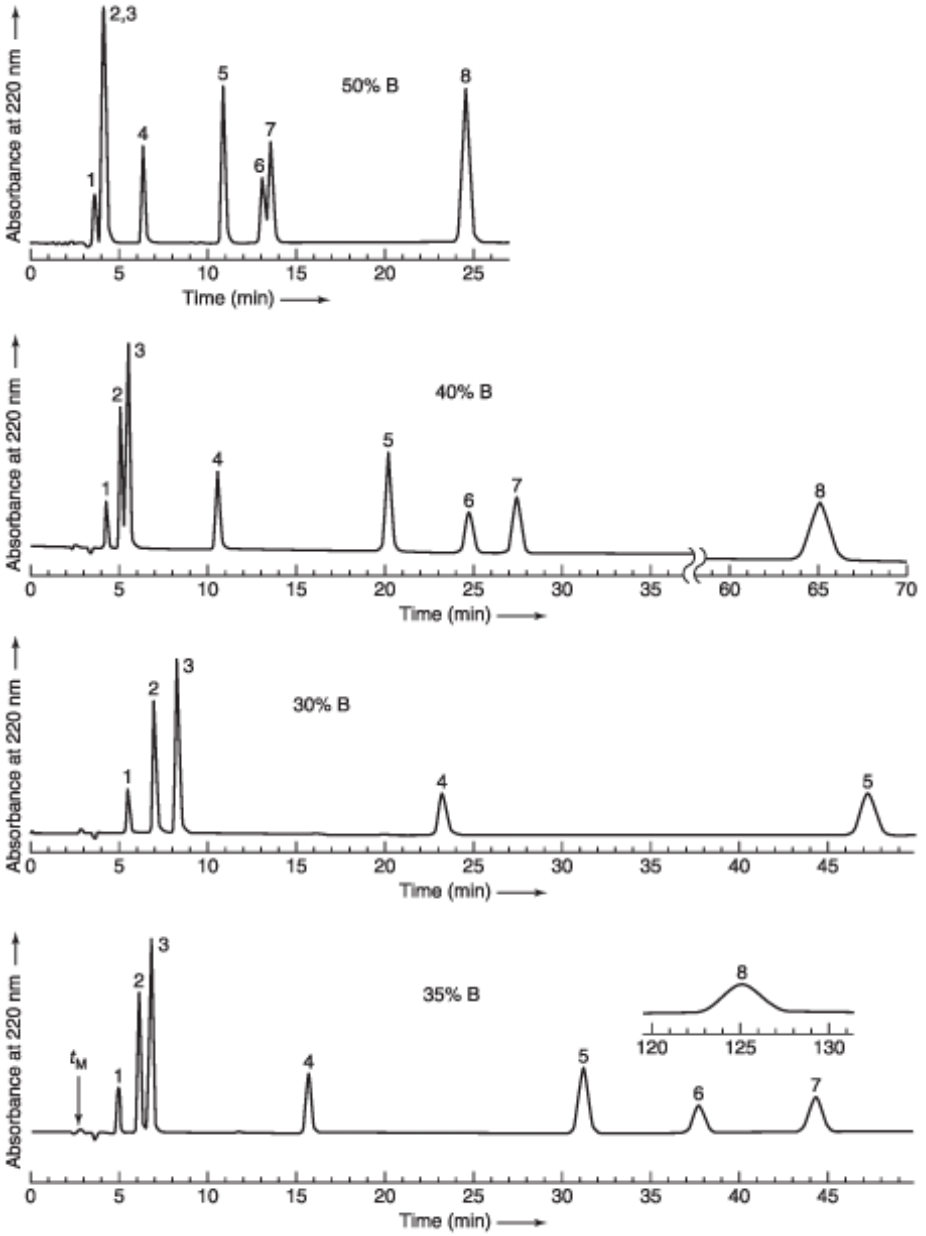
http://www.chem.agilent.com/Library/primers/Public/LC-Handbook-Complete-2.pdf

Change in Solvent Strength



- B = organic, RP separation
- Increasing organic reduces retention time – stronger solvent

Change in Solvent Strength



B=organic, RP separation

Impact of Increased % Organic

- Why reduced retention time?
  - More organic makes the MP more like the SP
  - Less drive of the analyte onto the SP

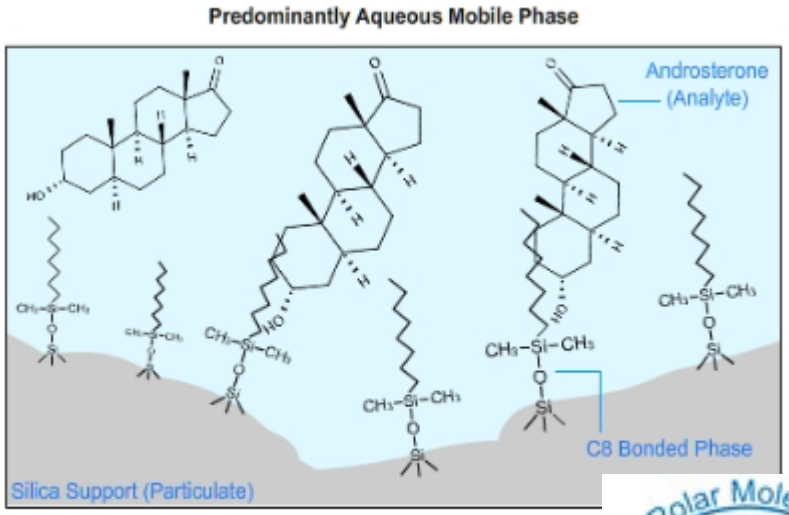
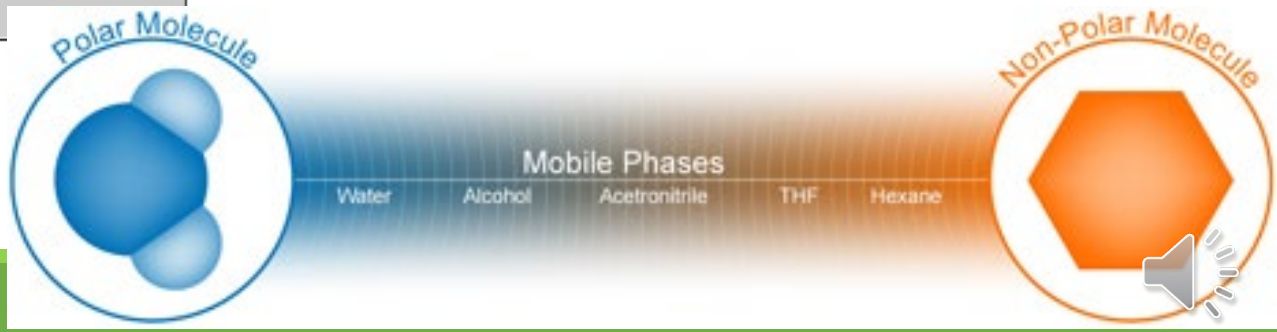


Figure 1: Schematic representation of Reversed Phase HPLC



Impact of Increased % Organic (RP)

- **ALL** compounds elute earlier

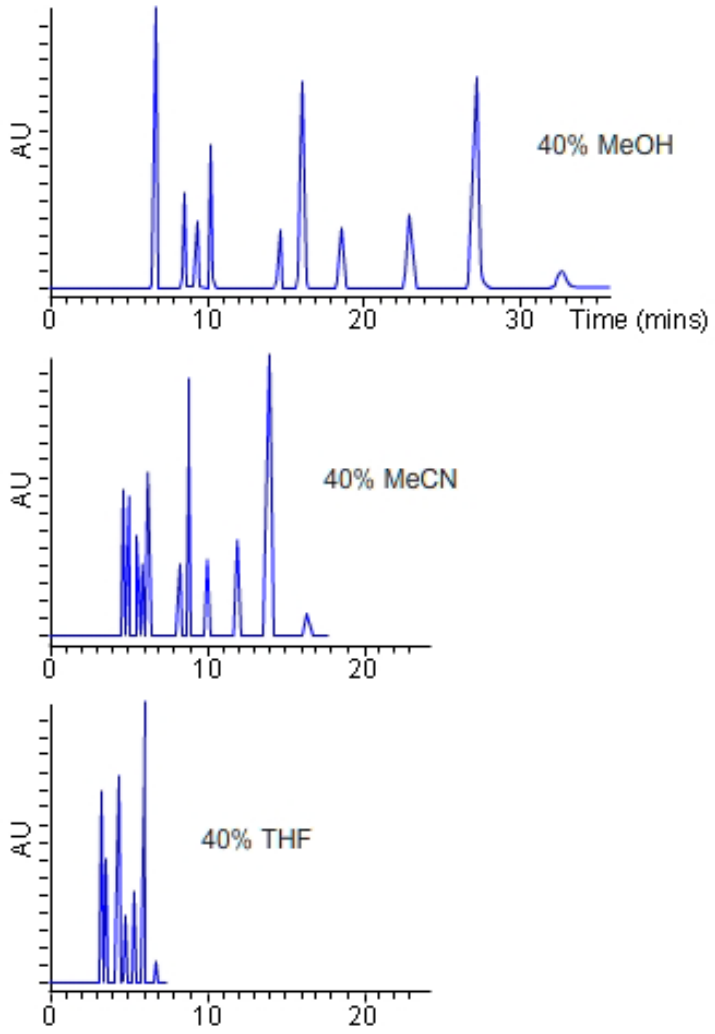
- Increase organic by 10% can decrease *k* by a factor of 2-3
- Typically a change of 5 to 15 % in retention time per 1 % change in organic composition

- This is a problem for reproducibility

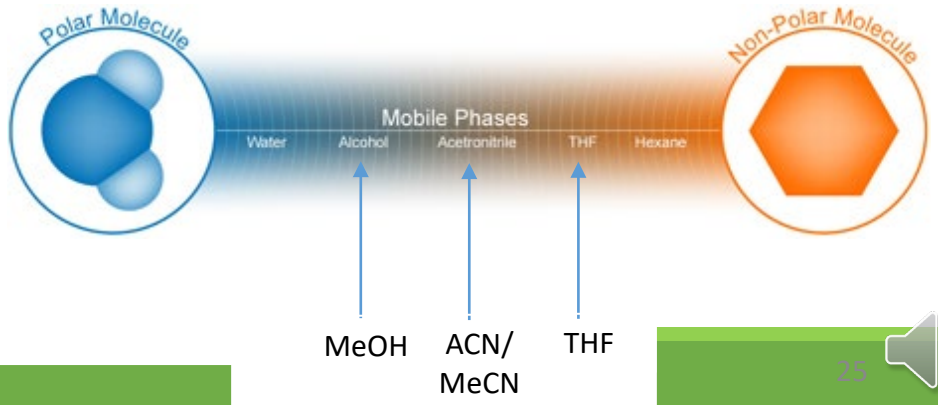
HPLC Simulator

- https://www.multidlc.org/hplcsim/4_2_0/
  - Look at the compounds benzylformamide, benzylalcohol, phenol, acetanilide, and phenylpropanol
  - Use water and ACN as the mobile phase with an isocratic method.
  - Change from 20%B to 22% B?
    - What happens?
  - Take a few minutes and play with the mobile phase conditions, the gradient options and the column properties - observe what happens to the chromatogram as you change these parameters

Impact of More Non-Polar MP



- Identical conditions, just ACN in lieu of MeOH
- ACN is less polar than MeOH
- ACN = stronger solvent
  - Moves things off the column faster



Impact of Stronger Solvent

- Why reduced retention time?
  - More non-polar organic makes the MP more like the SP
  - Less drive of the analyte onto the SP

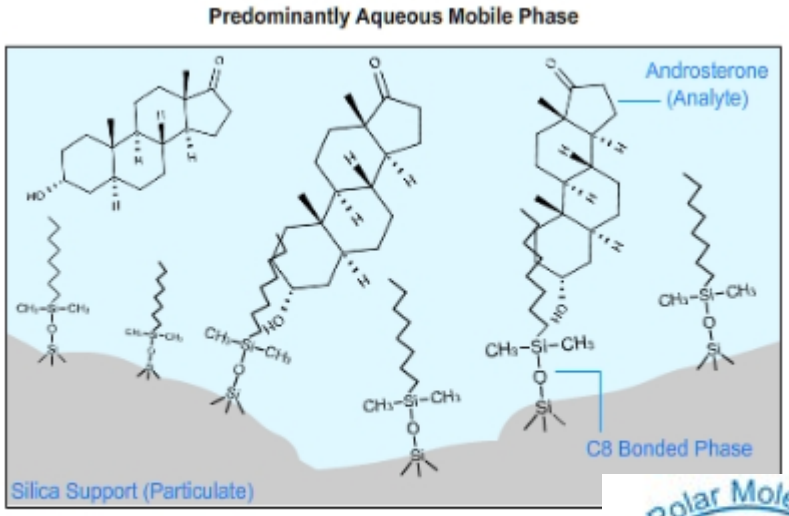
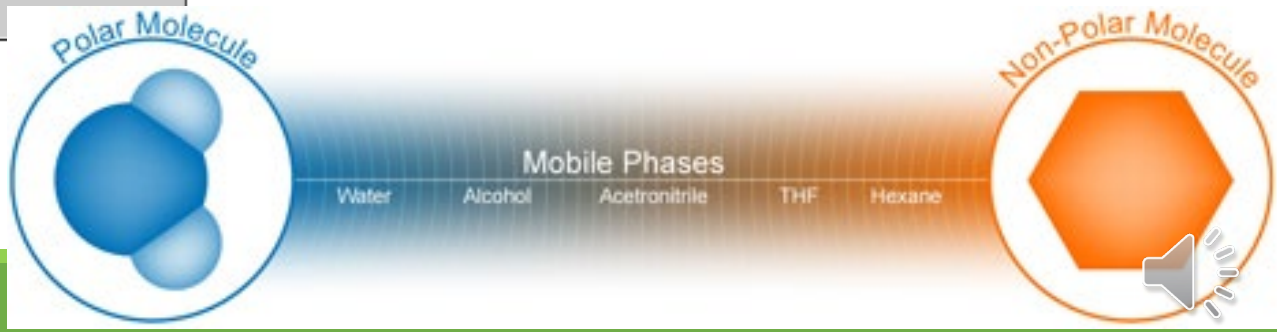
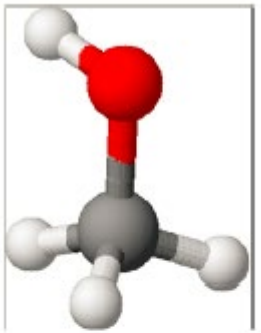


Figure 1: Schematic representation of Reversed Phase HPLC

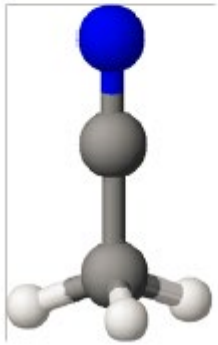


Organic Modifier

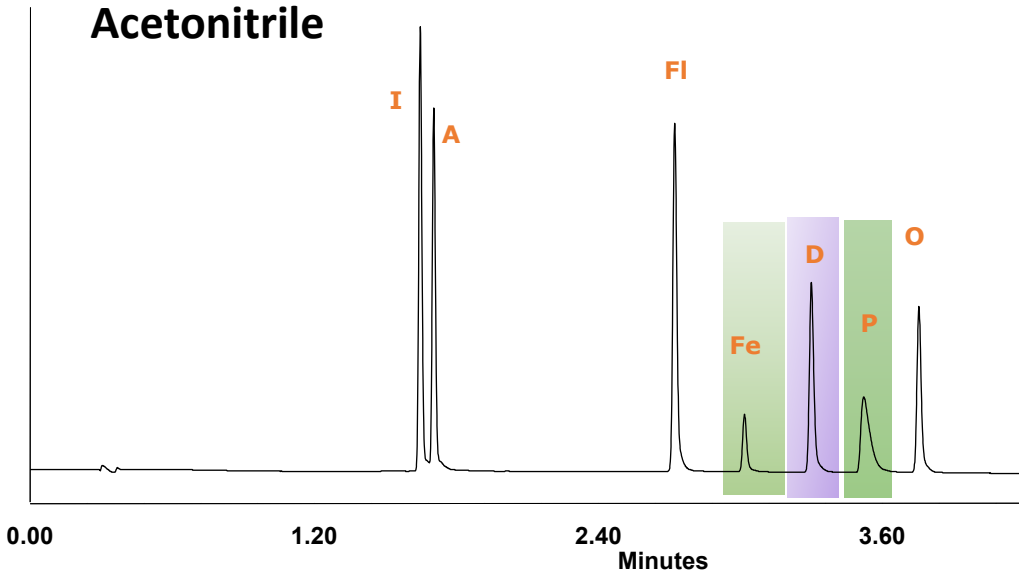
- Methanol
  - Protic solvent – hydrogen bond donor
  - Weaker elution solvent
  - Higher viscosity than acetonitrile



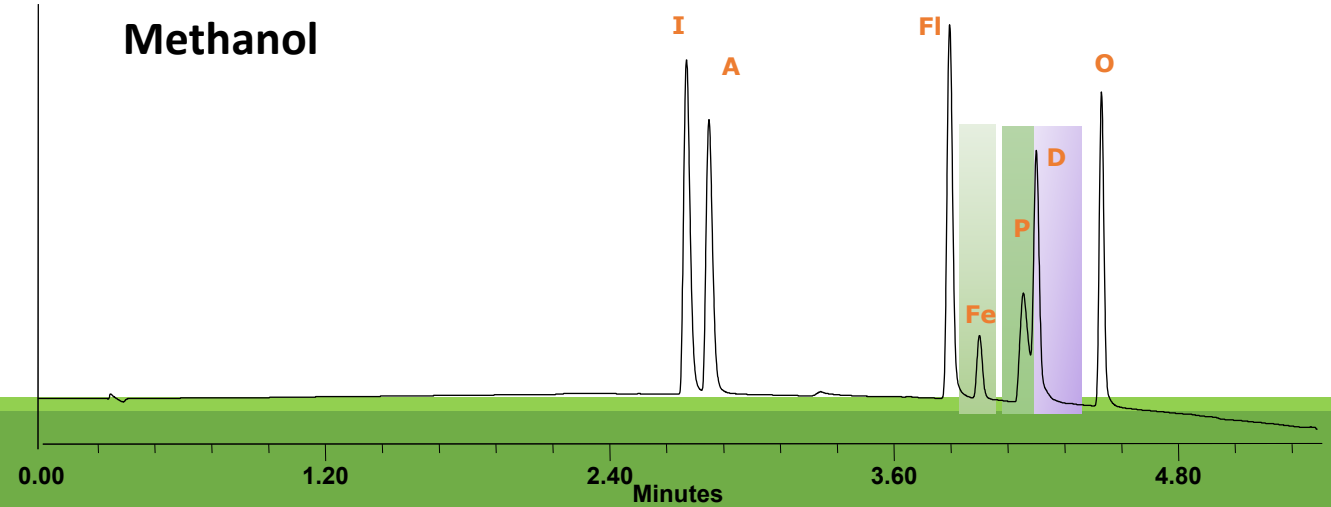
- Acetonitrile
  - Aprotic solvent – hydrogen bond acceptor
  - Strong elution solvent
  - Low viscosity



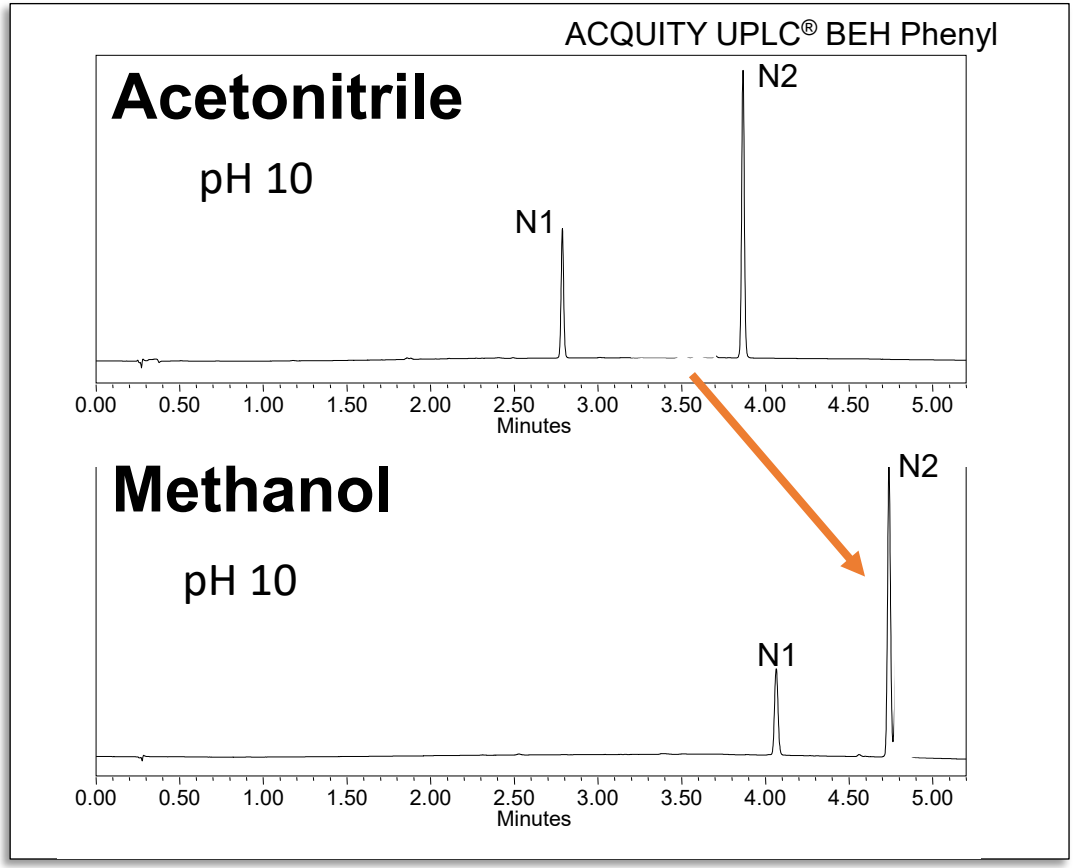
Changing Organic Modifier



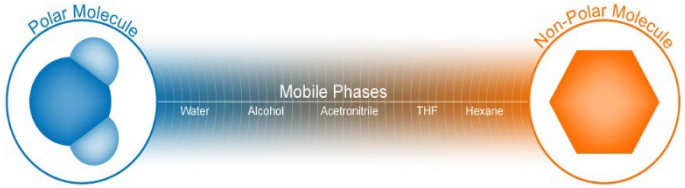
- Changing solvent or "organic modifier" can also change selectivity
- Must always confirm the elution order if make a change



RP Isocratic – ACN to MeOH



- MeOH is more polar than ACN → it is a weaker solvent
- Weaker = less able to compete with the stationary phase
  - Greater retention by reverse phase



- May also be some selectivity differences

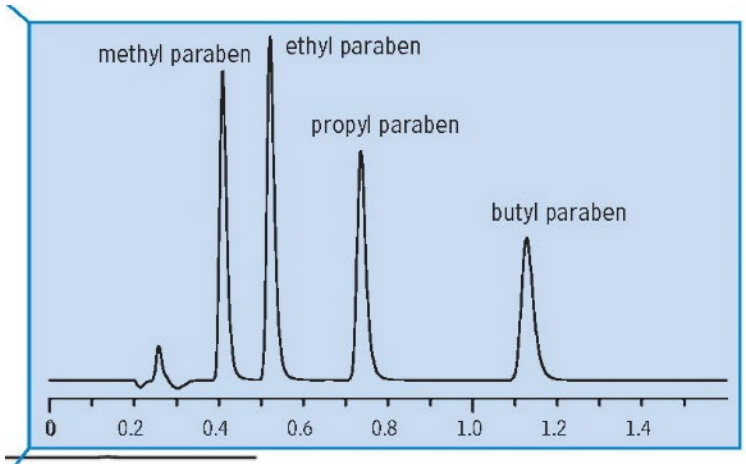
Images courtesy of Waters Ltd.

Impact of Organic

- Can use this knowledge to
  - Develop or modify methods
    - Elute faster or differently
  - Troubleshoot unexpected results

Troubleshooting

- You are working in A240 running the parabens experiment, your method calls for 70:30 MeOH:water and you expect this elution order and time. Instead you find
  - The elution time for all peaks is later than expected, what should you check?

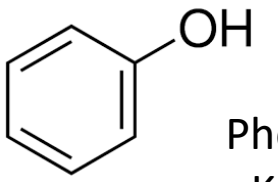


Troubleshooting

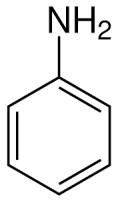
- You are running a published method using 50:50 water:MeOH
- The method worked yesterday (!) but now you find that most of your peaks are eluting earlier AND two have swapped places in the elution order
- What should you check/try?

Protonation State

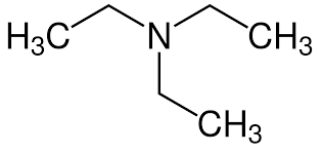
- Predict the protonation states of the following at pH = 4, 7, and 10



Phenol
pKa = 10

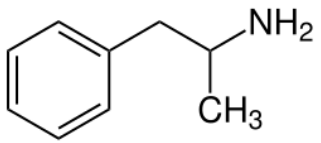


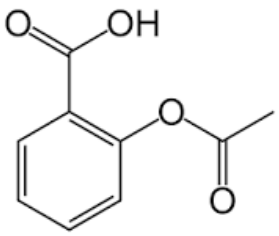
Aniline
pKa = 10



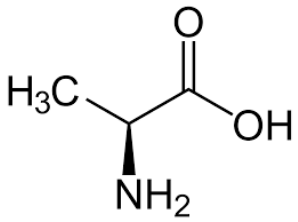
Triethylamine
pKa = 10.75

amphetamine
pKa = 9.9

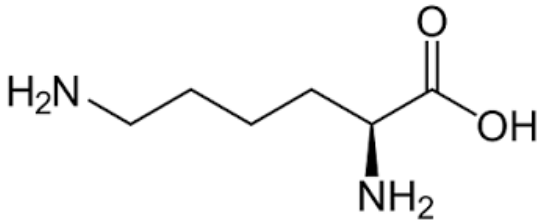




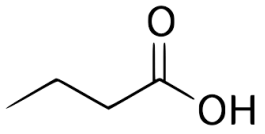
ASA
pKa = 3.49



Alanine
pKa-COOH = 2.34
pKa-NH3 = 9.69



Lysine
pKa COOH = 2.16
pKa-NH3 = 9.06
pKa – side chain NH3 = 10.54



Butanolic acid
pKa = 4.82

Impact of Mobile Phase pH

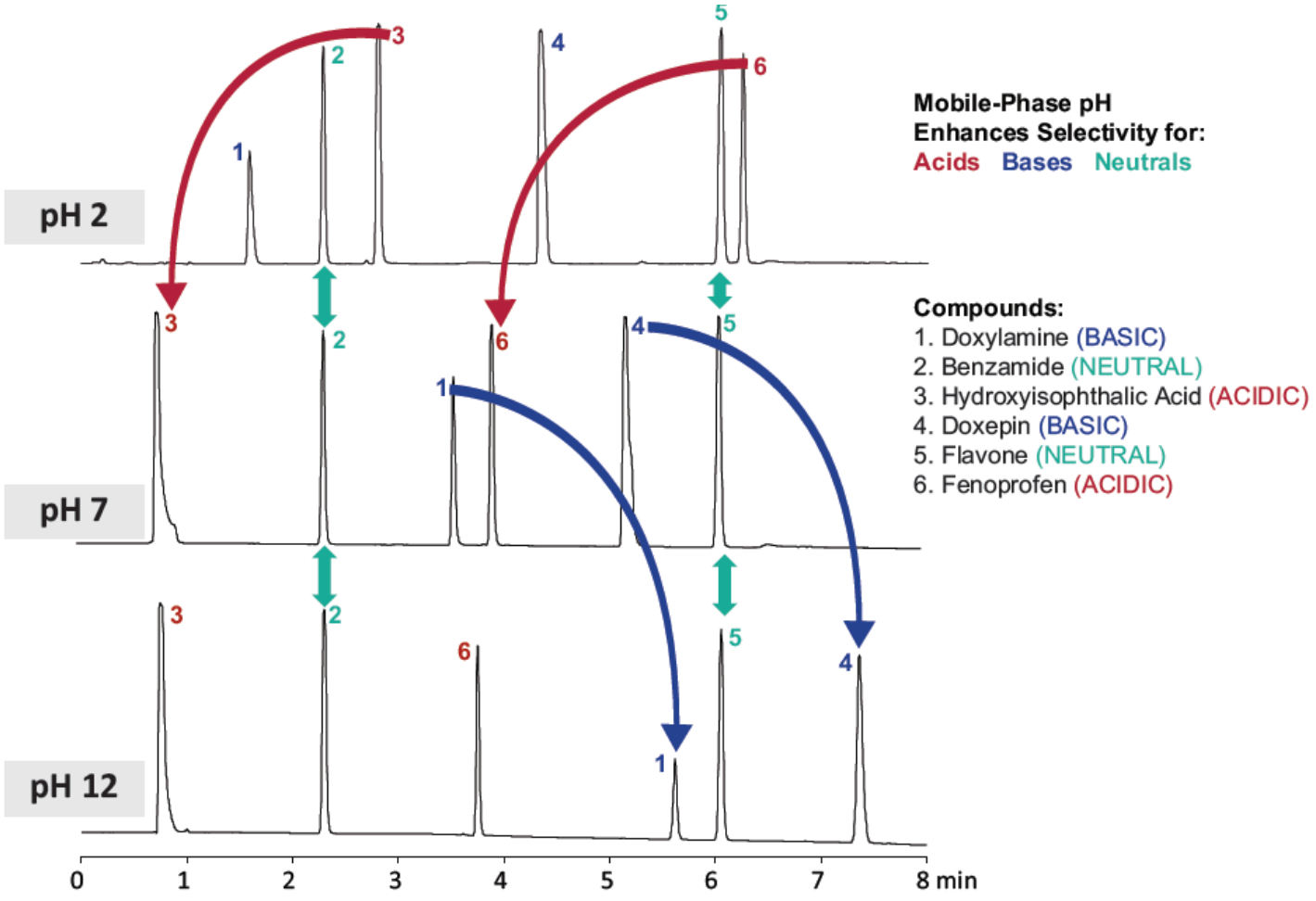
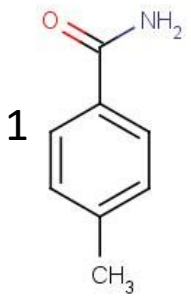


Figure courtesy of Agilent

Impact of pH on Retention

- Compounds:
  - p-Toluamide – neutral
  - Lidocaine – base, pKa 7.8
  - Ibuprofen – acid, pka 5.2



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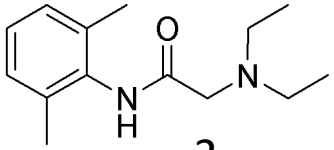

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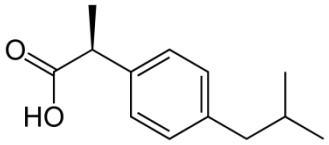

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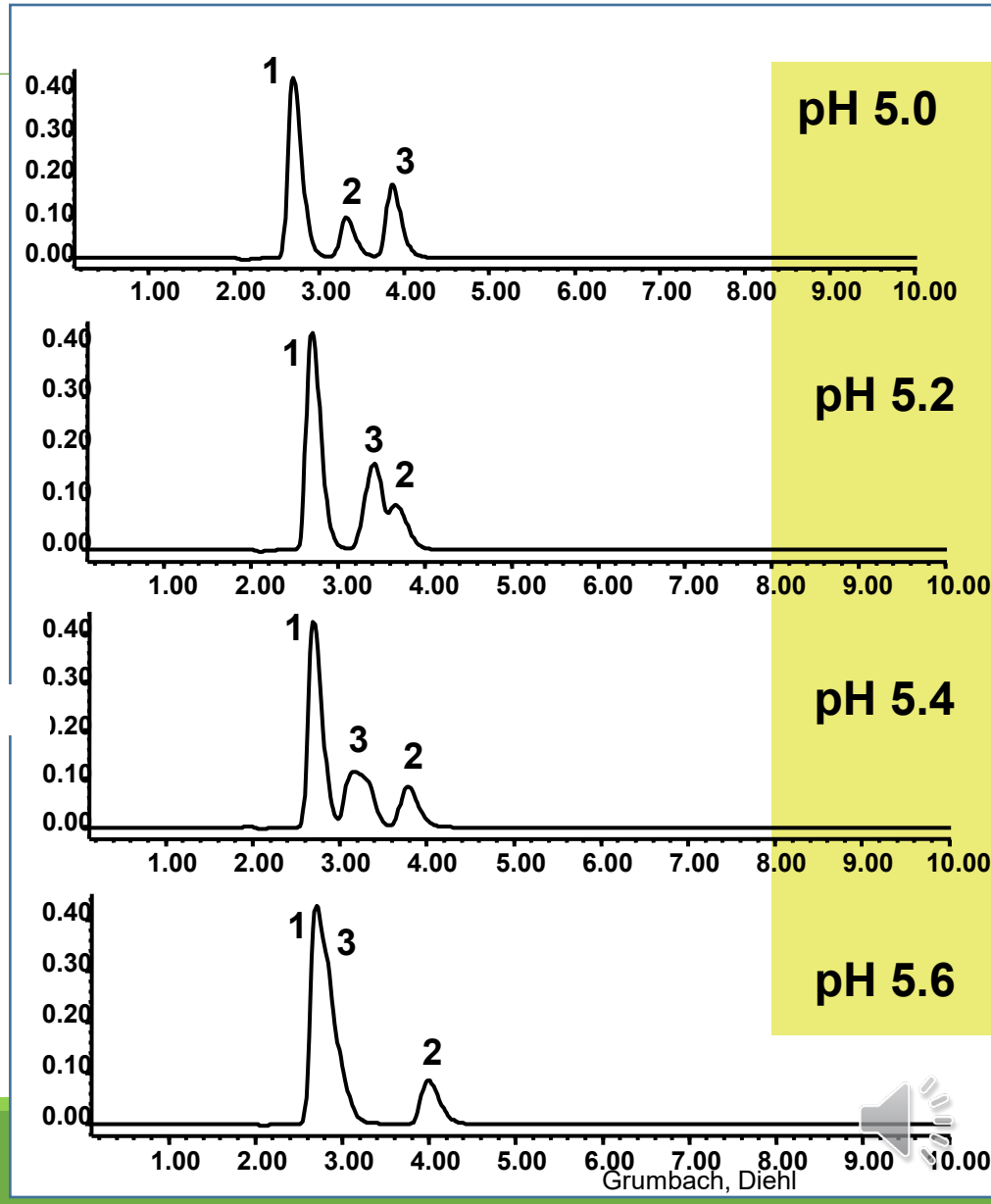
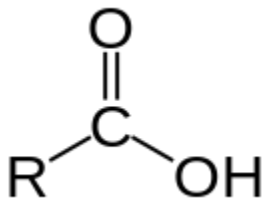


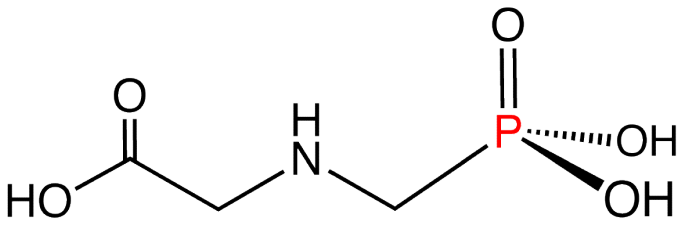
Figure courtesy of Waters

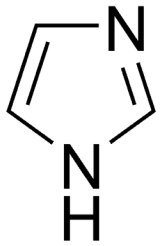
Grumbach, Diehl

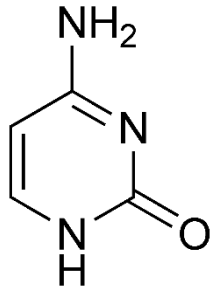
Mobile Phase: Buffers

- When would we use a buffered mobile phase?
  - When pH is important
  - What types of molecules would be involved – Ionizable or un-ionizable?
    - Ionizable

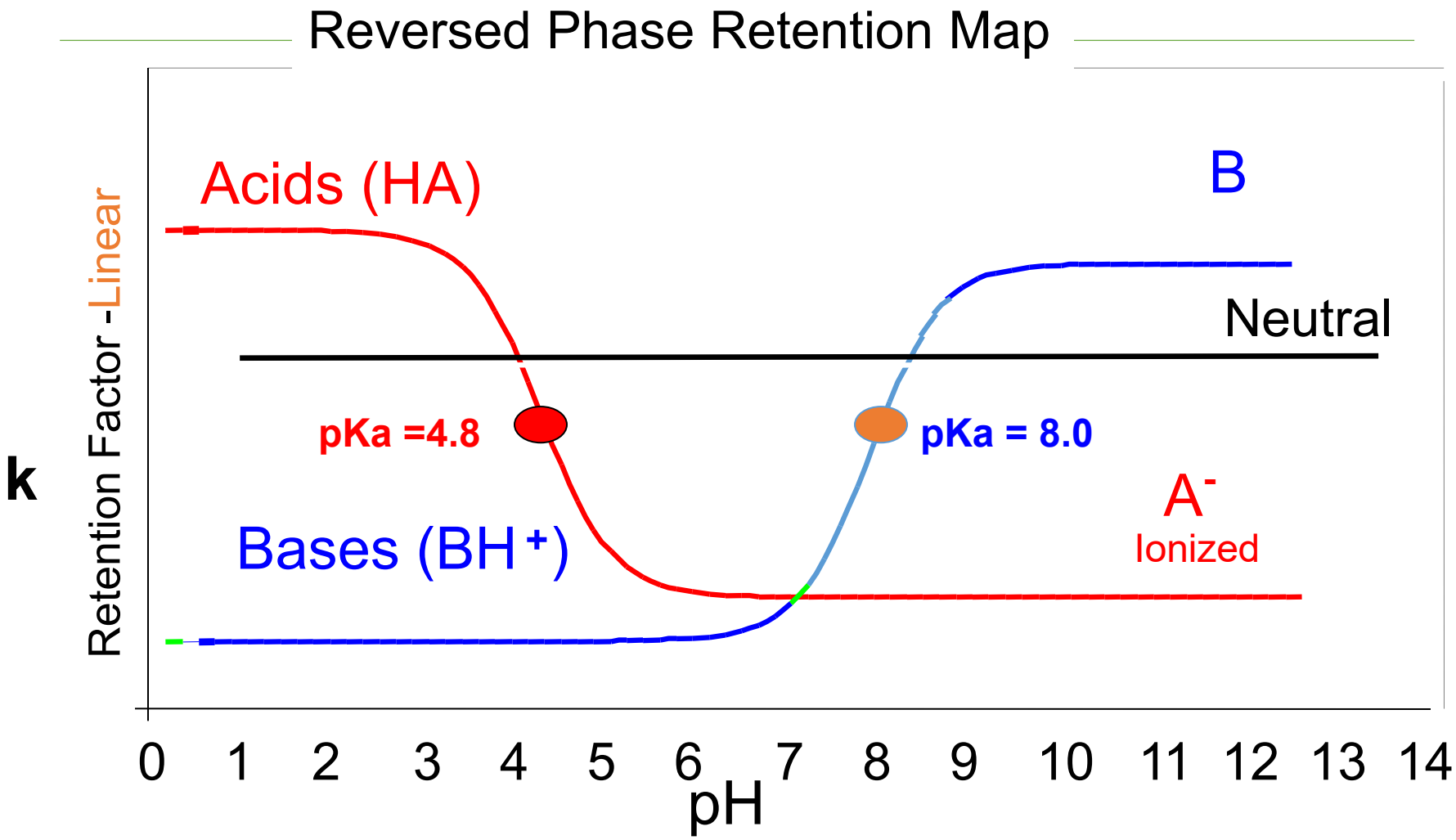




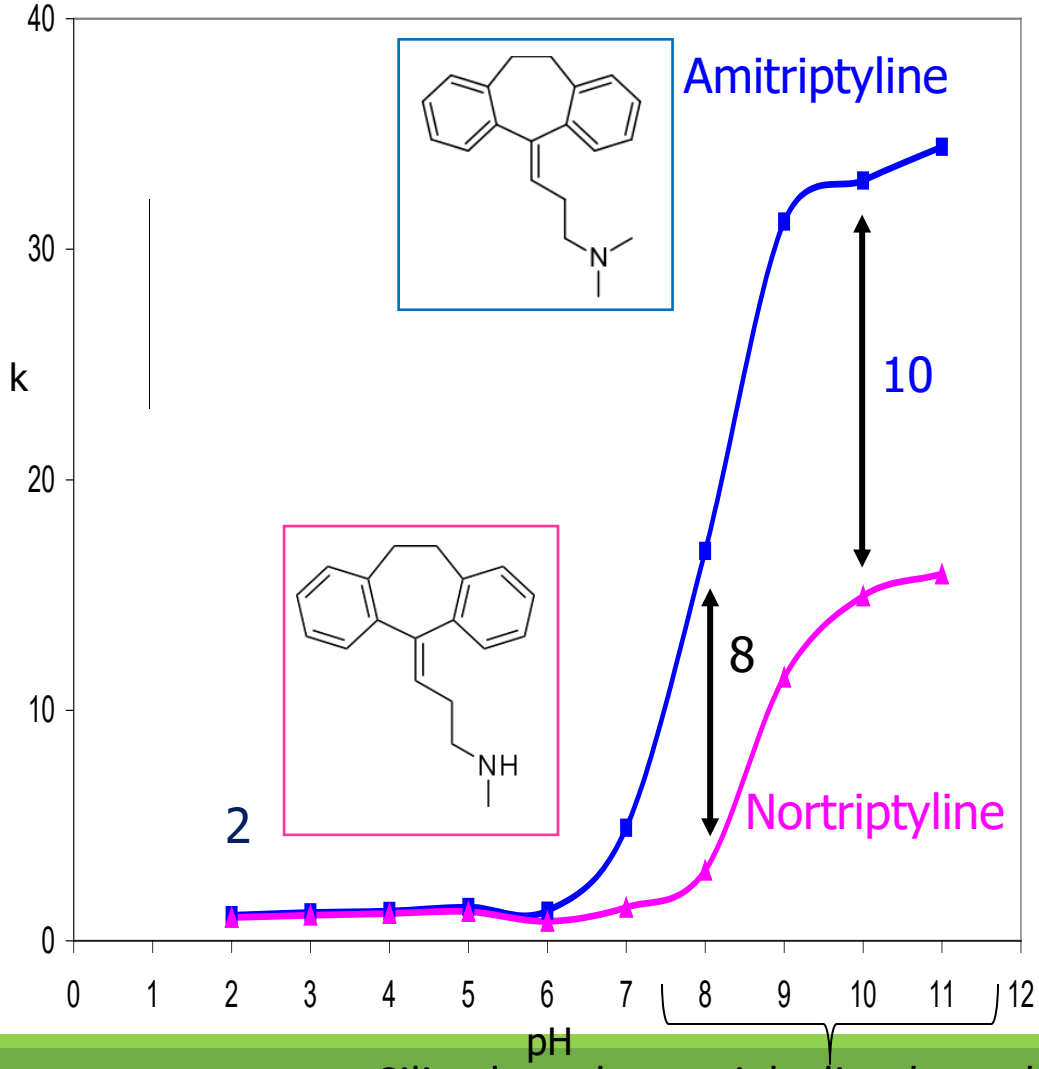


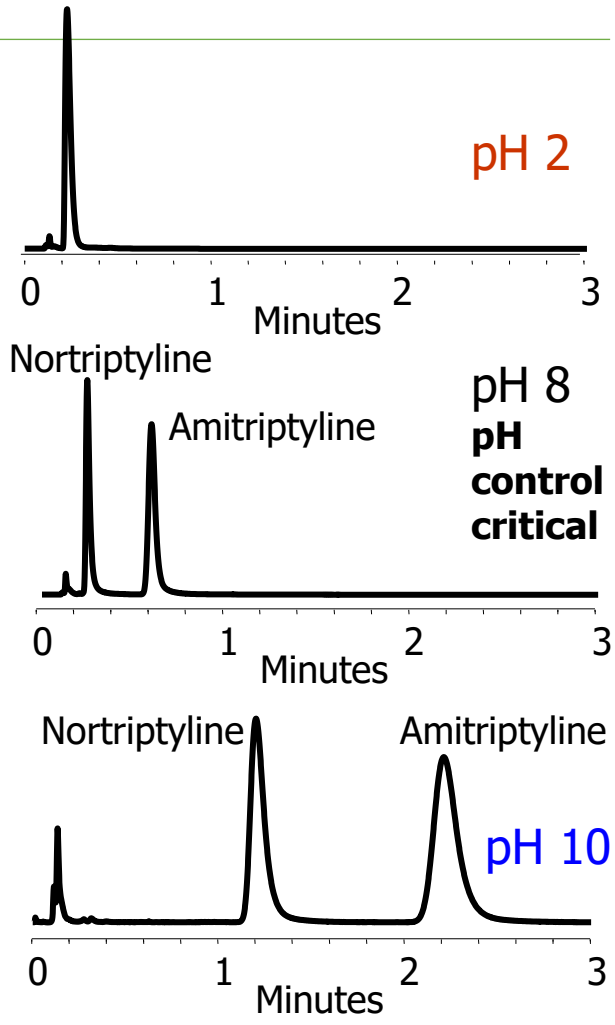


Effect of pH on Retention



Effect of pH on Retention





Silica-based materials dissolve at high pH

Consistency and pH

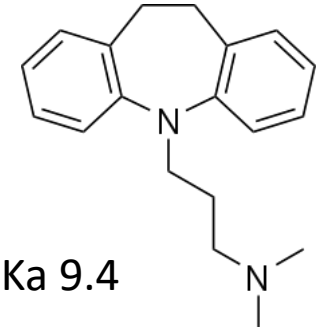
- Need to control pH to ensure consistent results
  - Especially if near the pKa of the analyte
- Ideally want to be 2 pH units from the pKa

Are Buffers Always Used?

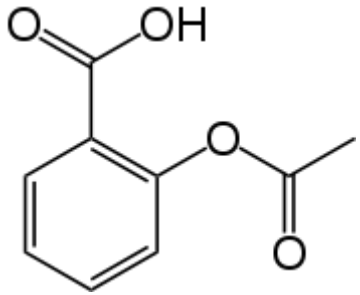
- No
- In some cases Trifluoroacetic acid (TFA) or formic acid (FA) are added in small amounts (0.1% v/v) to the mobile phase
  - Pull the pH down, assumed that exact pH not a factor

Reverse Phase and pH

- Some molecules can have multiple charged states



pKa 9.4



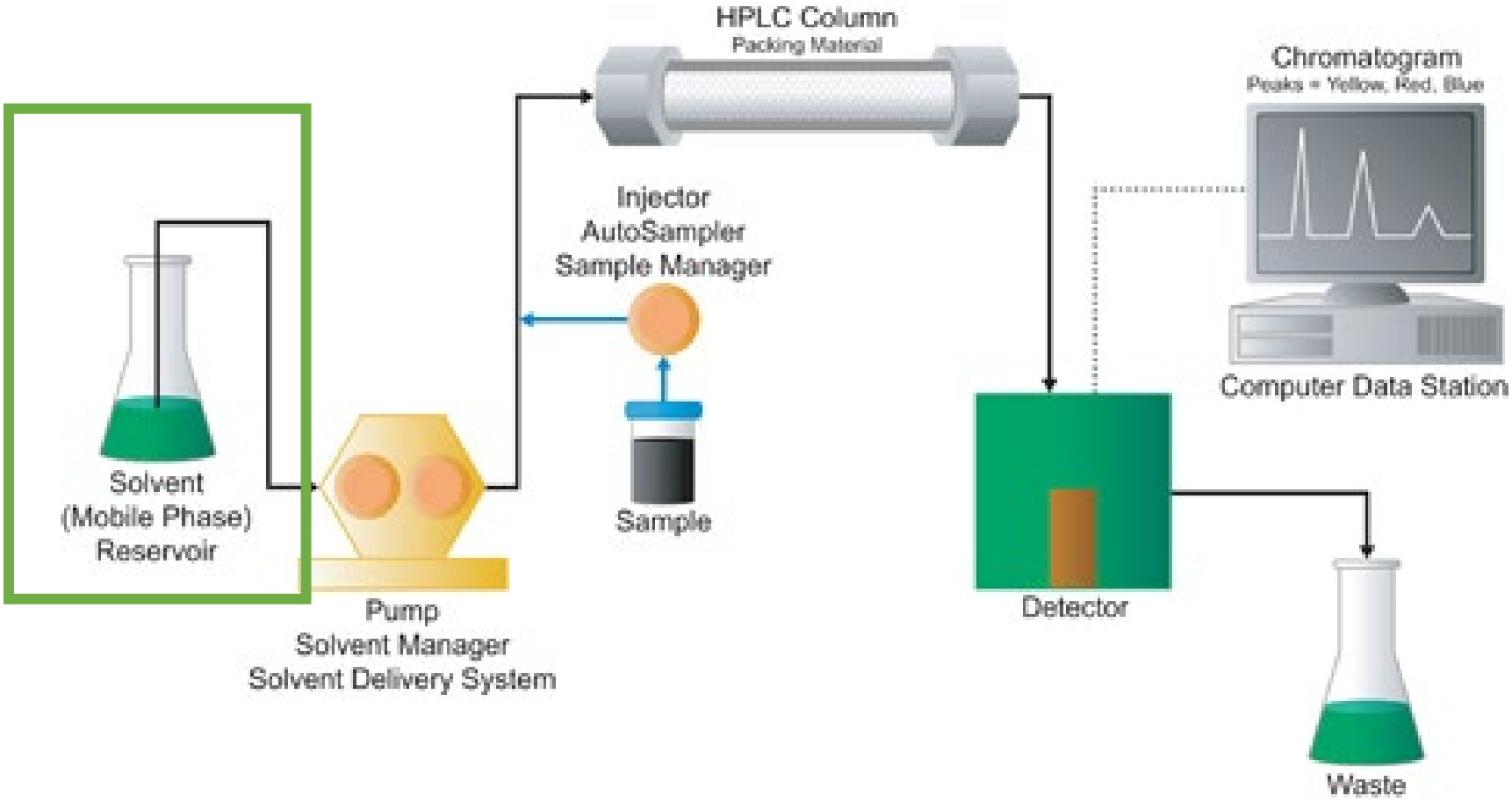
pKa 3.5

- Reverse phase methods use a non-polar surface
- What is will retain better by RP?
  - Charged or uncharged?
- How can we keep it in one state or another?
  - Through controlling the pH of the mobile phase

Troubleshooting

- Your solution to be analyzed contains
  - An amine (pka = 9) and other un-ionizable species
- Your conditions
  - 50:50 MeOH:pH 11 buffer (aq)
- You run your analysis and have excellent separation
- The next day you make up new mobile phases and find that the amine has reduced retention but all other peaks are unchanged.
  - What might be the problem?

High Performance Liquid Chromatography



Courtesy of Waters Canada

USP: Liquid Chromatography

From General Chapter <621>

The term "liquid chromatography" (LC), as used in the compendia, is synonymous with high-pressure liquid chromatography and high-performance liquid chromatography.

LC is a separation technique based on a solid stationary phase and a liquid mobile phase.

USP: Mobile Phase

From General Chapter <621>

The mobile phase is a solvent or a mixture of solvents, as defined in the individual monograph.

USP – Mobile Phase Examples

Mobile phase can be simple:

Acetaminophen Tablets:

Assay is HPLC based, Mobile phase is

- Solution A: 1% v/v glacial acetic acid in water
- Solution B: Methanol

How the mobile phase is prepared and stored impacts the results

Mobile phase can be more complicated:

Atorvastatin

Assay is HPLC based, Mobile phase is

- Buffer: 3.9 g/L of ammonium acetate in water. Adjust with glacial acetic acid to a pH of 5.0 ± 0.1.
- Solution A: Acetonitrile, stabilizer-free tetrahydrofuran, and *Buffer* (21:12:67)
- Solution B: Acetonitrile, stabilizer-free tetrahydrofuran, and *Buffer* (61:12:27)

The Mobile Phase

- Issues with mobile phase cause up to 70% of all LC problems
- What might go wrong with mobile phase?
  - Composition
  - Particulates
  - pH
  - Contaminates
  - Bacterial growth
- We must take great care in preparing, and documenting the preparation of, our mobile phase

Glassware

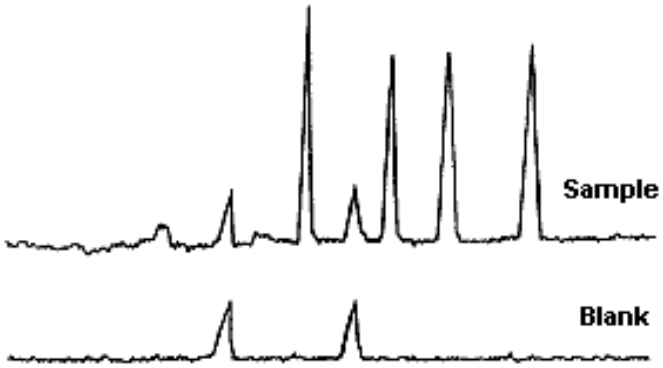
Choose your glassware wisely

- Only make up with clean glassware that has not been washed with detergent
  - Detergent can stick to glass and then leech off into the mobile phase, contaminating it
  - RINSE glassware with HPLC grade solvents and water only

https://mygoldfishisalive.com/why-do-my-goldfish-keep-dying

Impact of Detergent

- The detergent can then elute off the column during your gradient run causing an unknown or “ghost” peak



- What might be the implications if a ghost peak is seen in during a QC run for batch release testing?
  - Investigations, release delays, $$$$$$$$

Image courtesy of Sigma Aldrich

Solvent and Water Quality

- Choose the highest grade of solvent possible
  - HPLC Grade for HPLC work
  - Optima or similar grade for UPLC and/or MS work
- Choose the highest grade of water possible – Industry standard is ultrapure
  - Undergone successive steps of filtration and deionization & characterized as having a resistivity of 18.2 MΩ·cm at 25 °C
  - Commonly called “Milli-Q” water
    - Term a trademark of Millipore
    - Similar to Kleenex for tissue

Image courtesy of MilliPore

Filtration

- Everything that will enter a chromatographic system must be filtered
  - By you or by the manufacturer
  - This includes solvents, mobile phases and standards and samples in solution
- Filtration is happening continuously as the system is being used

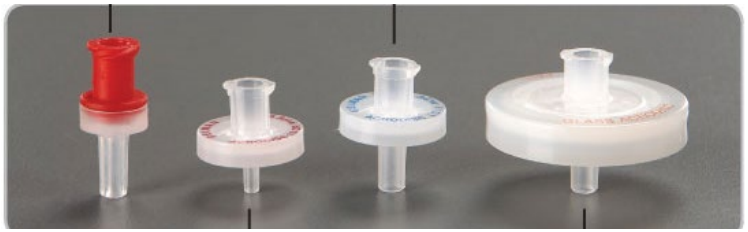
In-Line Filtration

- Performed on solvents and samples that have entered the system, prior to hitting the column
- Filters are placed
  - In the solvent, at the end of the solvent lines
  - In the pump, before the check valves
  - Between the pump and the injector
  - The injector and the column

Figure courtesy of Thermo Fisher

External Filtration

- On samples prior to injection
- Pore size of membrane filters
  - 0.45 um pore size for 3+ um particle size columns
  - Particle sizes <3 um use 0.22 um pore size

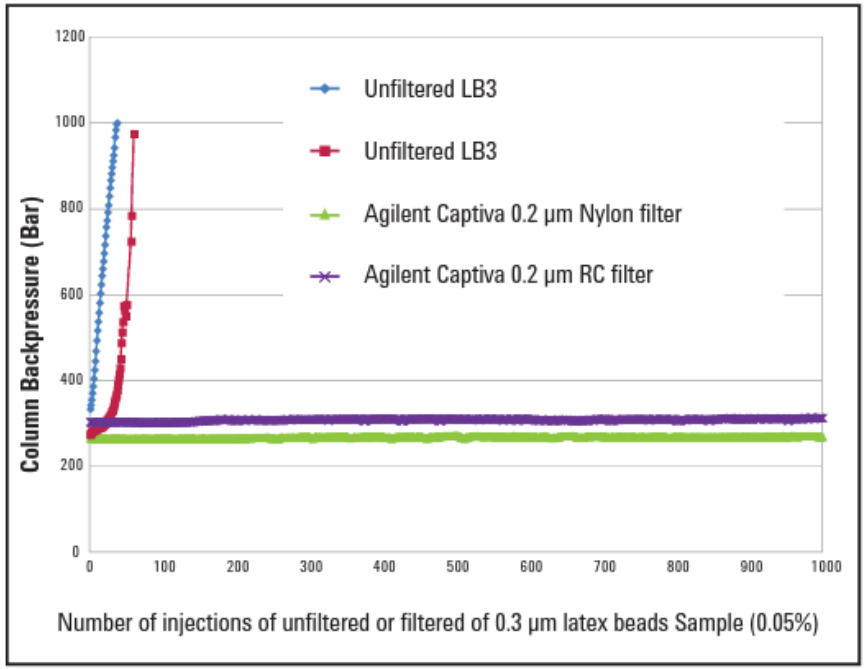


Images courtesy of Waters and VWR

Impact of Filtration

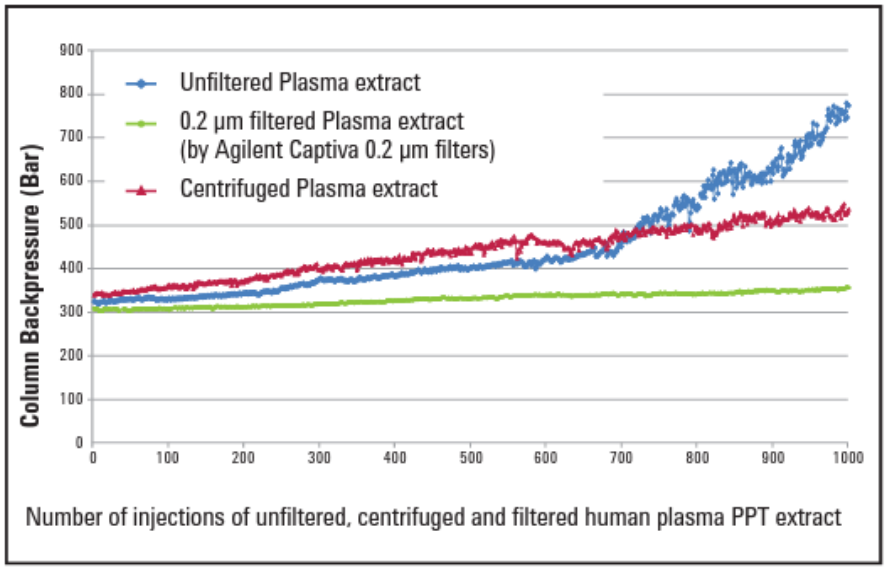
Results – Filtration impact on sub-2 micron column A by Latex Bead 0.3 µm solution

Effects on Filtration on sub-2 micron Column Life



Results – Filtration impact on sub-2 micron column B by Human Plasma PPT Extract

Effects of filtration on sub-2 column life time

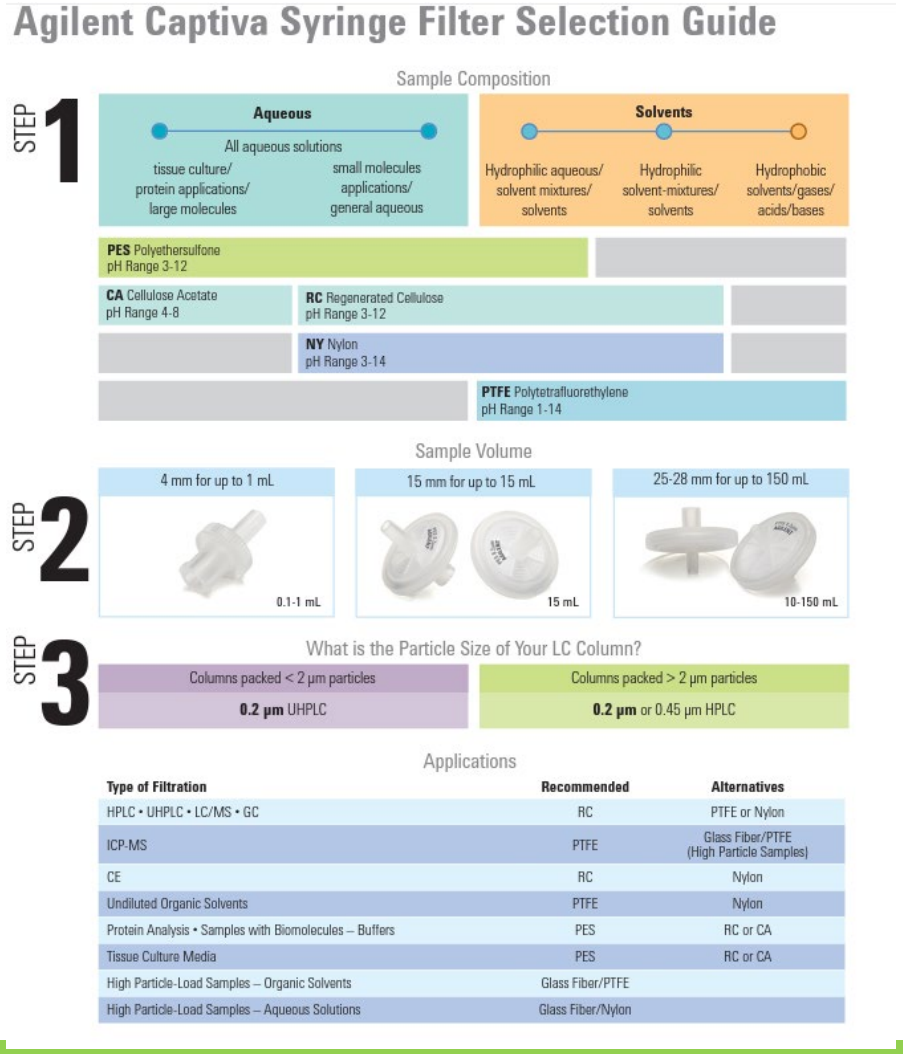


https://www.agilent.com/cs/library/brochures/5991-1230EN_Filtration%20Bro.pdf

Filter selection

- Filters are available in a variety of physical sizes and membrane types
- Filters must be chosen with their intended use/application in mind and must be used properly

Membrane Selection



Agilent Captiva Syringe Filter Selection Guide

STEP 1 — Sample Composition

Aqueous

All aqueous solutions

- tissue culture/ protein applications/ large molecules
- small molecules applications/ general aqueous

Solvents

- Hydrophilic aqueous/ solvent mixtures/ solvents
- Hydrophilic solvent-mixtures/ solvents
- Hydrophobic solvents/gases/ acids/bases

PES Polyethersulfone
pH Range 3-12

CA Cellulose Acetate
pH Range 4-8

RC Regenerated Cellulose
pH Range 3-12

NY Nylon
pH Range 3-14

PTFE Polytetrafluorethylene
pH Range 1-14

STEP 2 — Sample Volume

4 mm for up to 1 mL	15 mm for up to 15 mL	25-28 mm for up to 150 mL
0.1-1 mL	15 mL	10-150 mL

STEP 3 — What is the Particle Size of Your LC Column?

Columns packed < 2 µm particles	Columns packed > 2 µm particles
0.2 µm UHPLC	**0.2 µm** or 0.45 µm HPLC

Applications

Type of Filtration	Recommended	Alternatives
HPLC • UHPLC • LC/MS • GC	RC	PTFE or Nylon
ICP-MS	PTFE	Glass Fiber/PTFE (High Particle Samples)
CE	RC	Nylon
Undiluted Organic Solvents	PTFE	Nylon
Protein Analysis • Samples with Biomolecules – Buffers	PES	RC or CA
Tissue Culture Media	PES	RC or CA
High Particle-Load Samples – Organic Solvents	Glass Fiber/PTFE	
High Particle-Load Samples – Aqueous Solutions	Glass Fiber/Nylon	

- Defined by sample and solvent
- Choosing the wrong filter type can cause issues with
  - Solvent compatibility
  - Sample contamination
  - Sample loss

https://www.agilent.com/cs/library/brochures/5991-1230EN_Filtration%20Bro.pdf

External Filtration - Solvents

- Based upon application and mobile phase components
- Milli-Q water can be used directly
- HPLC and higher grades of solvents can typically be used from the bottle

External Filtration - Buffers

- Any time a solid is added to the solvent it must be filtered before being put on the system
- Remember: Buffers are used to control the pH of a solution
- All additives should be of the highest grade possible
- Must filter to remove particulates

Buffers - Salts

- Different buffers will control pH changes at different pH ranges
  - Related to their pKa values
- In older methods Na & K salts were very common as were phosphate buffers
  - BUT these are not amendable to MS
- Modern methods tend to move away from non-volatile buffers
  - Ammonium with acetate, formate and bicarbonate are now more common

Volatile Buffers

- Non-volatile buffers interfere with the performance of mass spectrometers
- LC/MS is becoming more and more prominent
  - Environmental – emerging contaminants
  - Biotech/biopharm – important for characterization
  - Pharma – impurity identification
  - Food – residue analysis
  - Useful for compounds without a chromophore
- Often labs without a MS develop amenable methods just in case…

Drawbacks of Filtration

- For **trace analyses** involving sensitive MS detectors filtration is often avoided
  - Can contribute contamination greater than analyte concentrations
- Often solid additives are avoided for MS work
  - Use high quality liquid additives

Diquat/Paraquat Recovery Data (n=7) for Three Types of Water Spiked at 40 ng/L

Sample Type	Recovery (%RSD) Diquat	Recovery (% RSD) Paraquat
Groundwater	82 (8)	89 (16)
Tapwater	84 (5)	88 (8)
River water	83 (4)	89 (19)

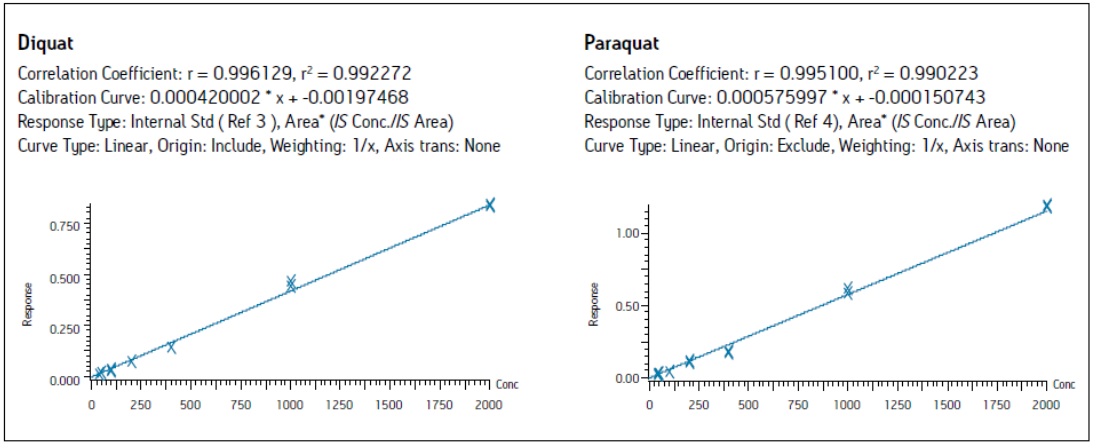


Figure 4. Typical LC/MS(MS) Calibration Curves.

http://www.waters.com/webassets/cms/library/docs/720004220en.pdf

Mobile Phase Preparation

- If we are making a mixed mobile phase (1:1 ACN: 10 mM sodium acetate, pH 4), when do we measure pH?
  - Before we add organic or after?
  - Remember: The presence of organic solvent in a solution affects the accuracy of a pH meter
  - The addition of organic solvents also changes the pH of a solution if not buffered
- Depending on the analytes, minor changes in pH can impact results
- Most modern methods mix solvent ON the system
  - Using line A and B

Kahoot

- Mobile Phase part 1

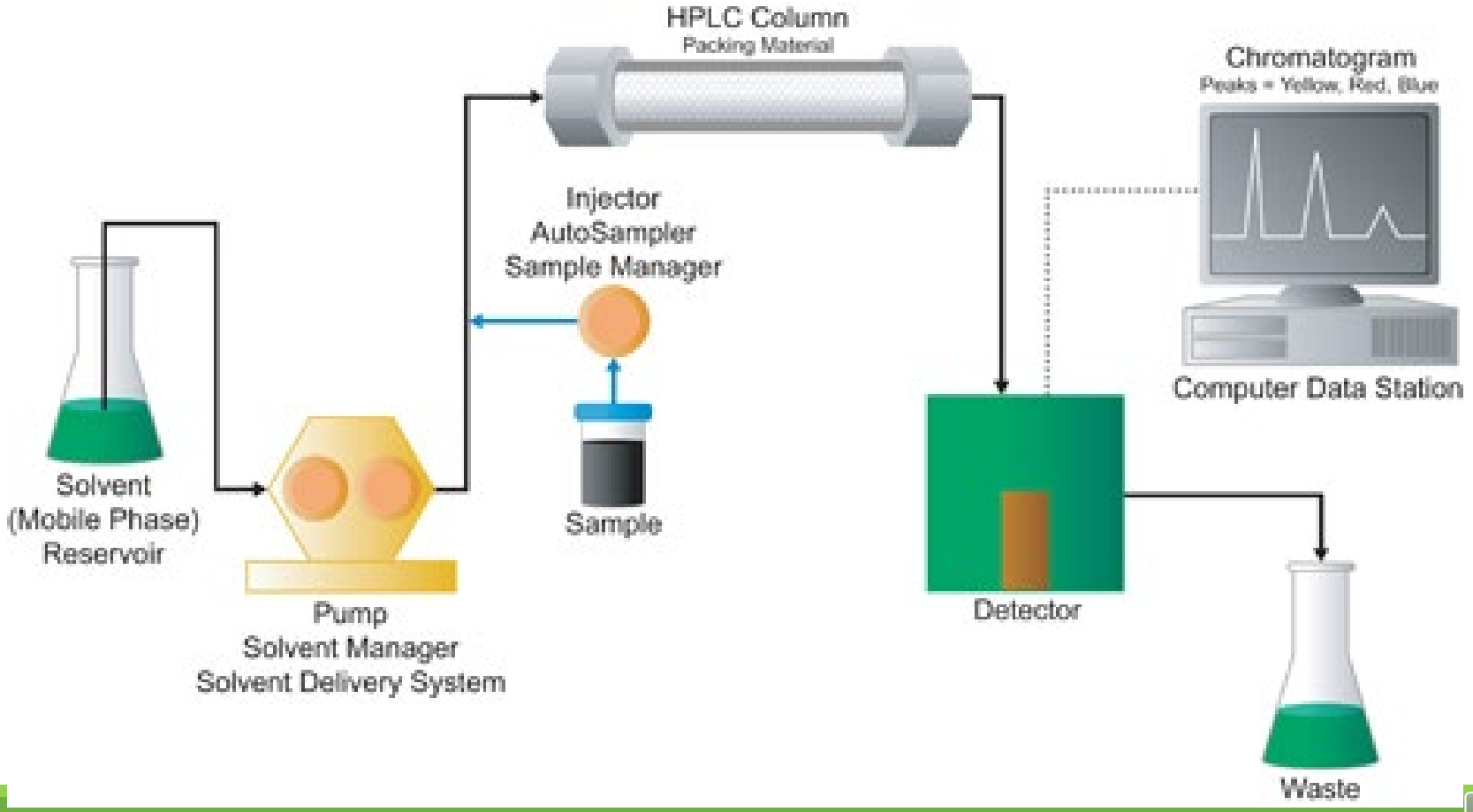
Mobile Phase - Degassing

- Solvents equilibrate with gases in the atmosphere and the solubility of gases in a pure solvent is higher than that of a mixture
  - When solvents are mixed together, the solubility decreases, the solutions are over saturated and gas bubbles form

https://www.forbes.com/sites/kalevleetaru/2017/12/18/why-was-2017-the-year-of-the-filter-bubble/?sh=319c4ebd746b

Mobile Phase - Degassing

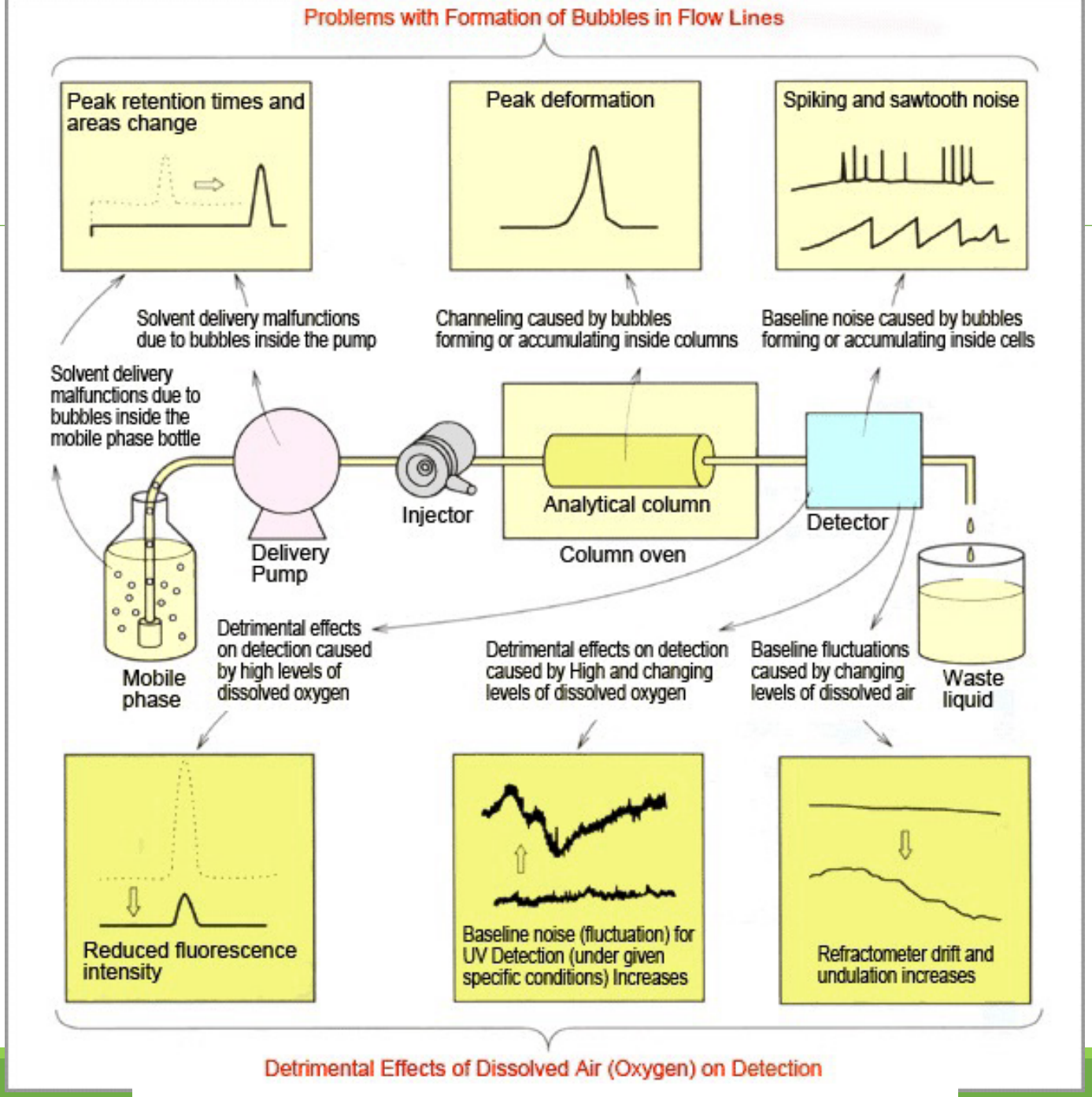
- When do solvents mix in HPLC?



Mobile Phase - Degassing

- Solvents equilibrate with gases in the atmosphere and the solubility of gases in a pure solvent is higher than that of a mixture
  - When solvents are mixed together, the solubility decreases, the solutions are over saturated and gas bubbles form
  - Gas bubbles can occur on mixing or anywhere within the system where a nucleation site exists

https://www.forbes.com/sites/kalevleetaru/2017/12/18/why-was-2017-the-year-of-the-filter-bubble/?sh=319c4ebd746b



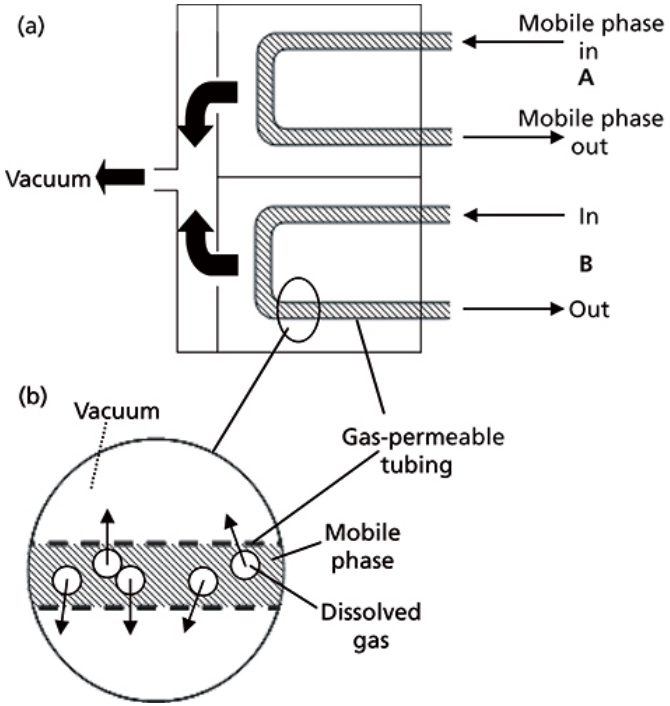
http://www.shimadzu.com/an/hplc/support/lib/lctalk/s5/01.html

Mobile Phase – Degassing Benefits

1. Baseline stability
   - Detector is free of bubbles – no sudden changes in signal & pump is consistent in solvent delivery
2. Injection volume reproducibility
   - No air in lines to affect the introduction of the band of sample in to the MP stream
3. Retention time reproducibility
   - Pump operation, solvent composition and sample introduction are consistent run to run

Mobile Phase – Degassing Methods

- Modern LC systems have built in degassers which are effective for most applications



http://www.chromatographyonline.com/mobile-phase-degassing-what-why-and-how

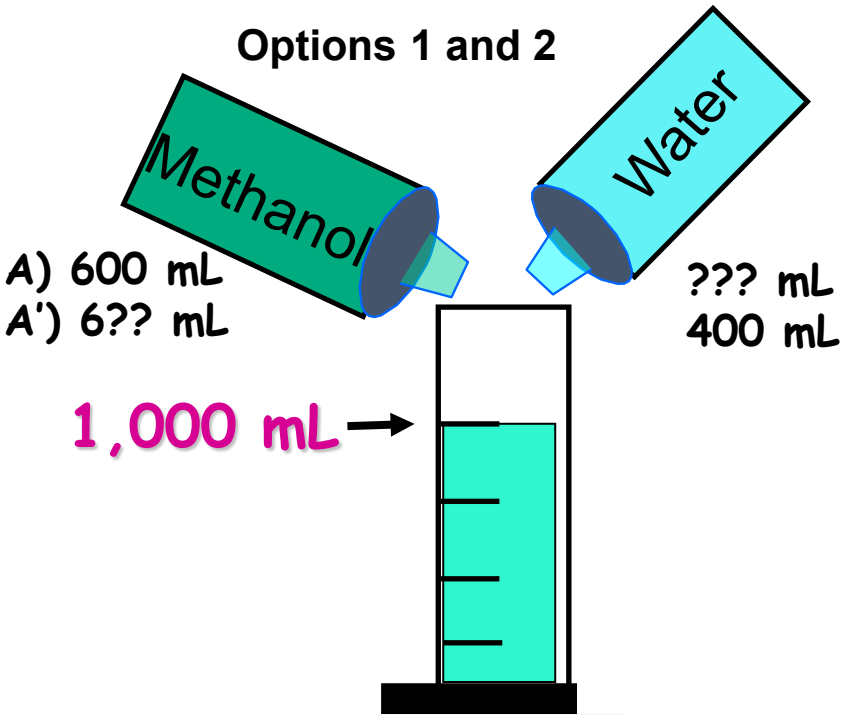
Mobile Phase – Mixed Solution

- Some methods will require solvent mixtures as the mobile phases
  - Particularly older methods
- Examples
  - A = 95% ACN, 5 % water; B = 1:1 ACN:water
  - A = water, B = 90% MeOH, 10% THF
- How the solution is made is critical for success

Mobile Phase – Mixed Solvent Prep

- How would you prepare 1L of a 60:40 MeOH:Water solution?
  1. Measure out 600 mL of MeOH in a graduated cylinder and fill to 1L with water
  2. Measure out 400 mL of water in a graduated cylinder and fill to 1L with MeOH
  3. Measure each separately and combine
  4. Weigh each out separately and combine

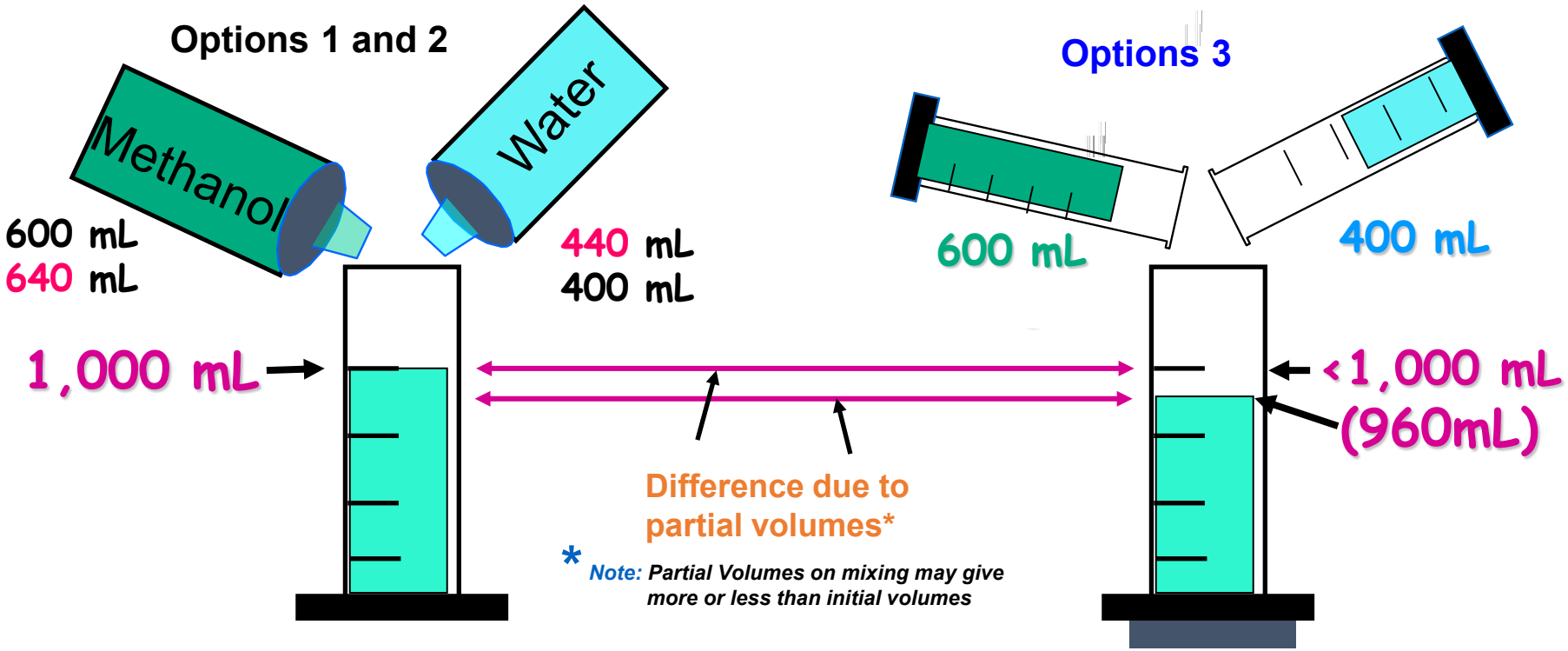
Mobile Phase – 60:40 Mix Prep



Options 1 and 2: Adding organic/aqueous directly to measuring vessel

Need to specify which solvent is added first: e.g. H_2O to MeOH or **MeOH to H_2O**

Mobile Phase – 60:40 Mix Prep



Options 1 and 2: Adding organic/aqueous directly to measuring vessel

Need to specify which solvent is added first: e.g. MeOH to H_2O or H_2O to MeOH

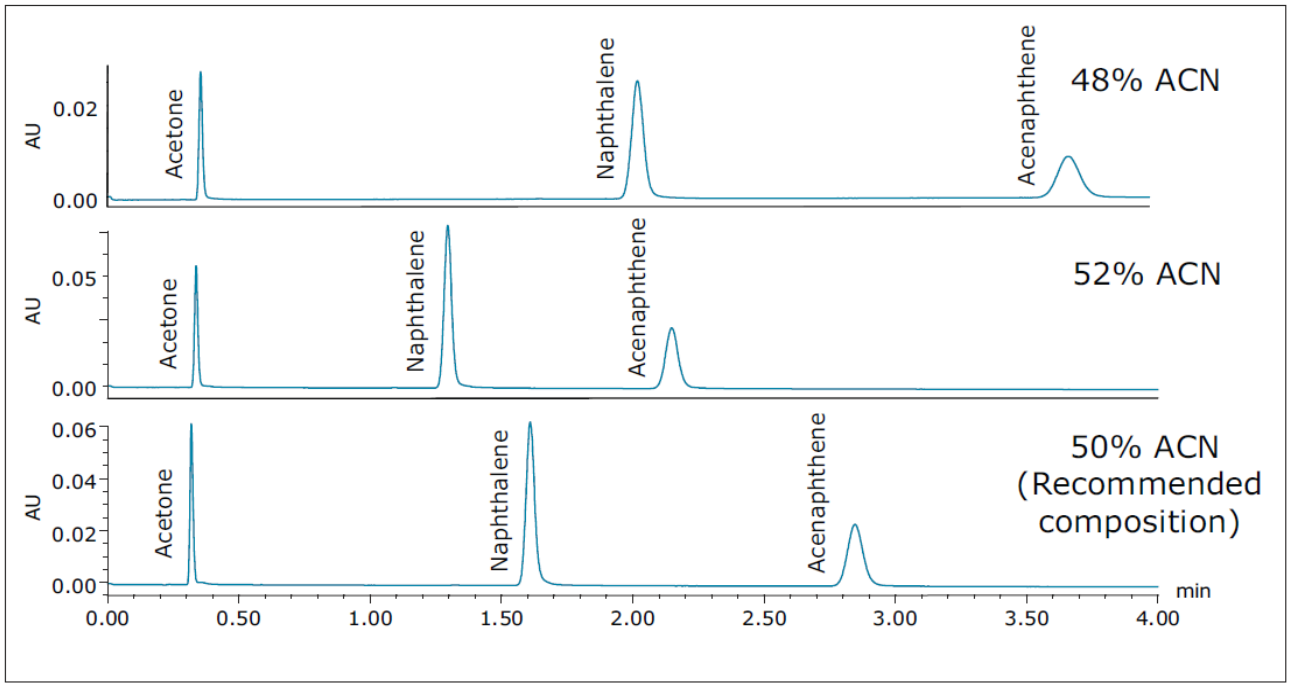
Option 3: Separate volumetric measurement **preferred method**

Mobile Phase – 60:40 Mix Prep

- Measuring out the solvents separately THEN mixing is the preferred method
  - Volumetrically is most common
  - Weighing is most accurate
    - Eliminates any volume differences due to temperature
    - Rarely done
- You must clearly state how your mobile phase is prepared

Impact of Improper Mixture

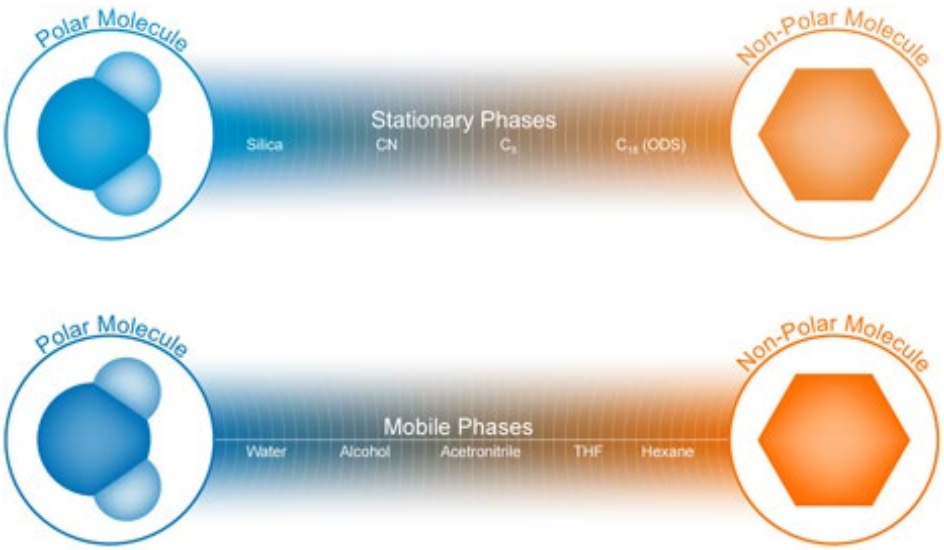
- In reverse phase, what is the impact of too much organic? (ACN or MeOH)
  - Things elute too fast



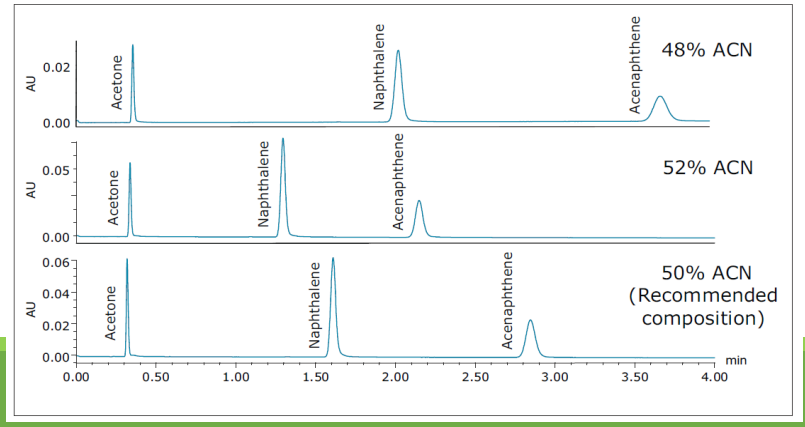
Images courtesy of Waters Canada

Remember Strong vs Weak Solvents

- Strong solvents are more like the stationary phase
  - Push analytes off faster
  - Better able to compete for analytes



- Weak solvents are different from the stationary phase
  - All analytes will move slower



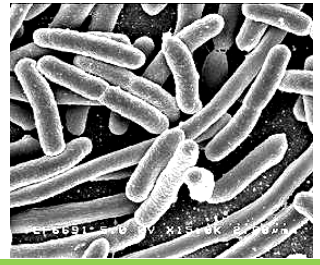
Mobile Phase -Storage

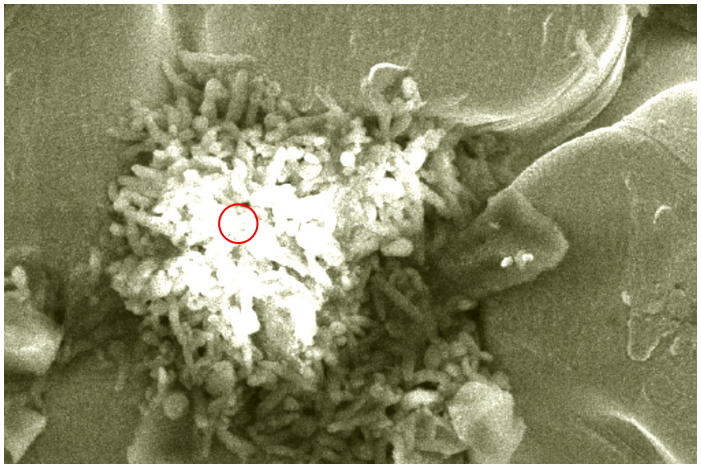
- Mobile phase should be covered and insulated to help ensure that its intended characteristics are maintained
- What might change?
  - Composition
  - Contaminants
- MP's containing buffers and water have a finite life.
  - Should be discarded daily or every other day
  - Why?
    - Grow microbes

Mobile Phase - Storage

- Aqueous solutions should be made every day or every other day
  - Why?
  - Aqueous solutions will grow algae and bacteria which will enter the system and clog lines and columns
    - Over pressure and/or peak shape problems

Scanning Electron Microscopy (SEM) Images of Column Frits

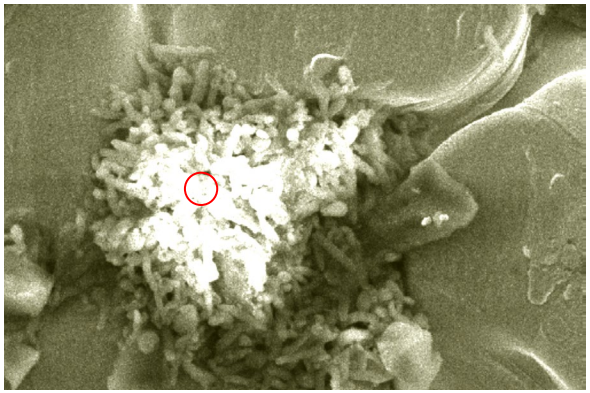




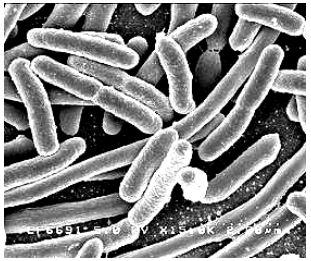
Images courtesy of Waters Canada

Mobile Phase – Microbial Growth

- Microbial growth causes
  - Blockages in the filters, frits, columns and pump
  - Ghost peaks
    - Especially during gradient elution
  - Increased background noise
- Mobile phases should always be labeled and dated
  - When made and expiration date



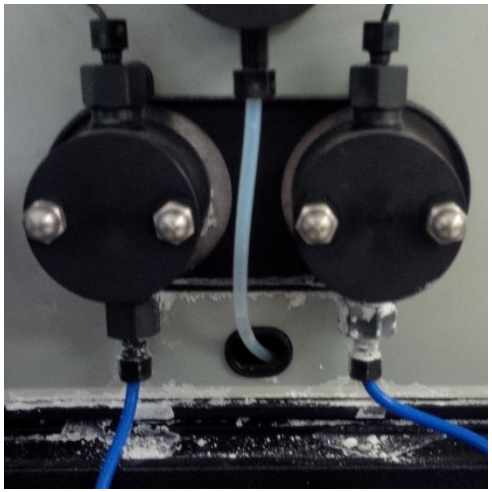
Scanning Electron Microscopy (SEM) Images of Column Frits



Images courtesy of Waters Canada

Microbes in Mobile Phase

- To prevent microbial growth in an idle HPLC system
  - Flush buffers out with water
  - Store the system in minimum of 10% organic (MeOH or ACN)
- Buffers must be flushed from system after use
  - May precipitate and create significant blockage as well as degradation of the piston seals (seals fall apart)



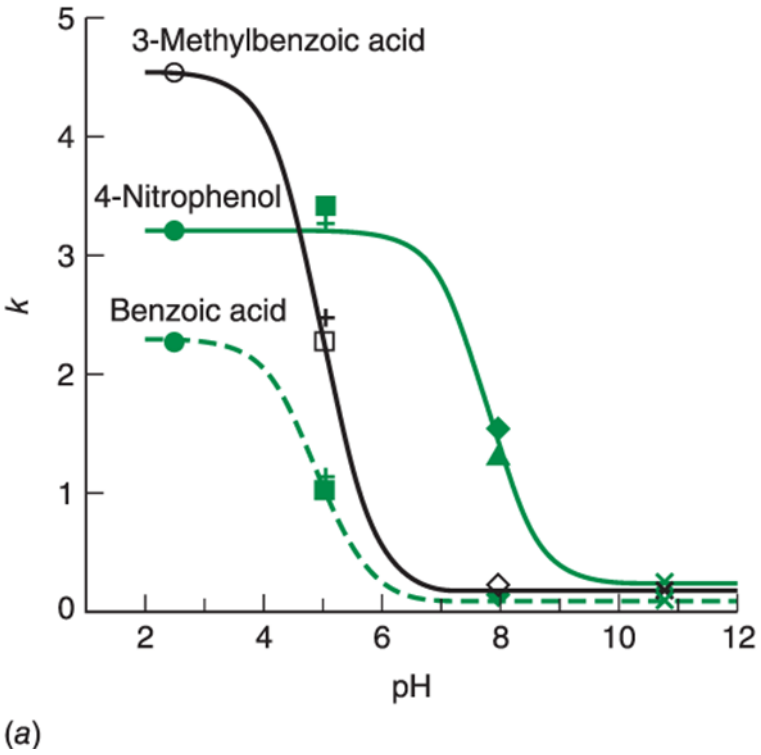
Mobile Phase - Storage

- Ensure sealed to avoid evaporative loss
  - Affects composition
  - Make up fresh every day or every other day
- Do NOT use Parafilm
  - Dissolves in organic solvents
  - Bleeds into solvent
  - Builds up on the head of the column and elutes as a blob-o-gram
- All mobile phase should be labelled
  - When made, by who, what it is, when it expires

Image: https://www.sporeslab.io/shop/Parafilm-PM992-Laboratory-Tape-p307692608

Kahoot

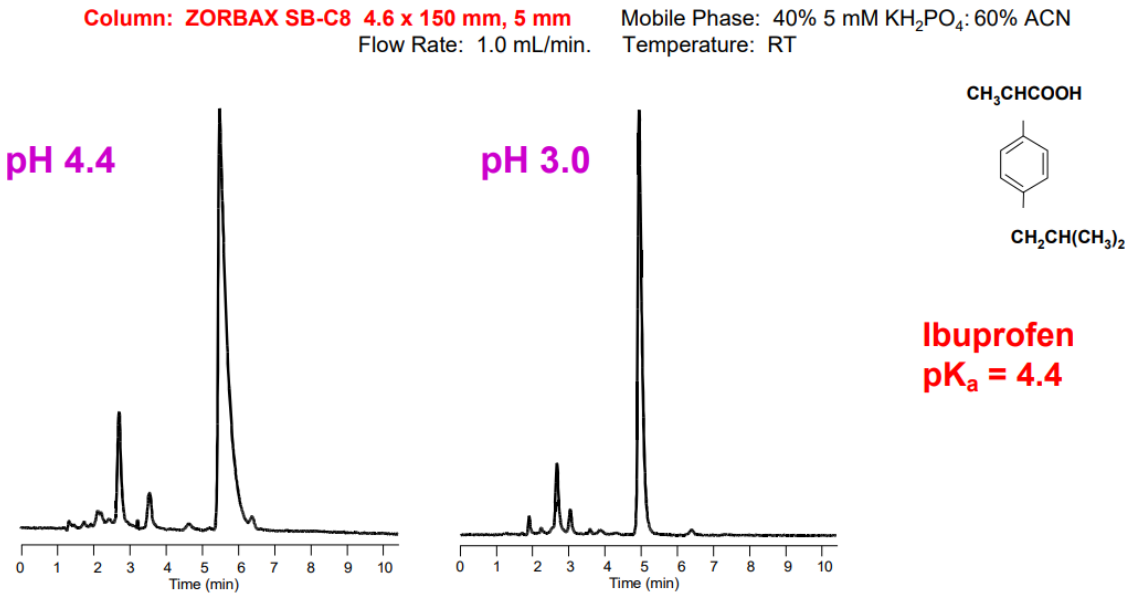
Practice Problems



(a)

- You are provided the data to the left.
- If you were to develop a method, what pH would you choose to have retention of all three compounds with robustness?
  - Why?
- What would you add to the mobile phase to help with robustness?
- What key things would you need to do to ensure uptime for the instrument when preparing and storing the mobile phase?

Practice Problems

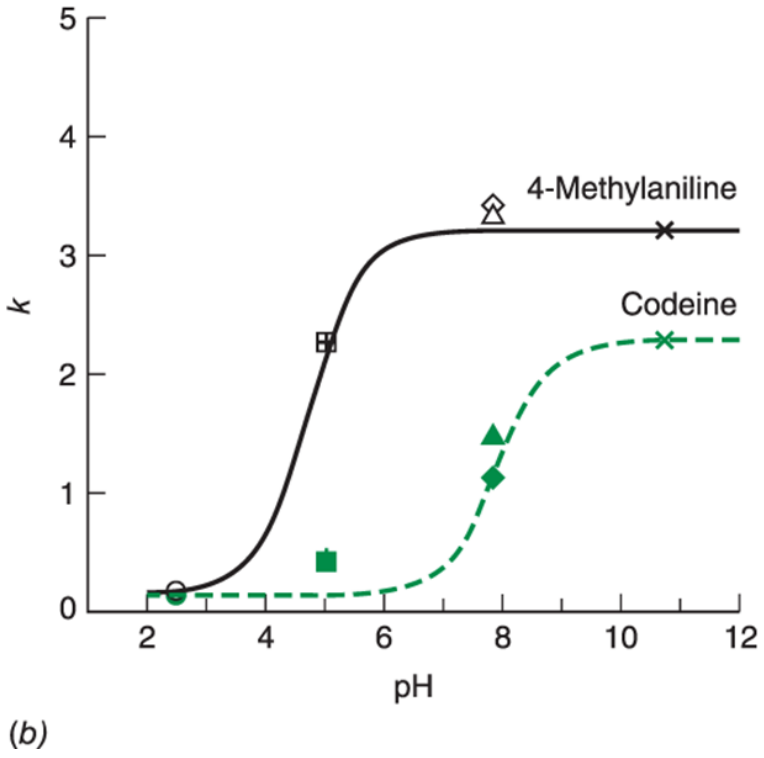


- Explain the difference between pH 4.4 and pH 3.

https://www.agilent.com/cs/library/posters/public/Final%20TIPS%20and%20Tricks%20HPLC%20Troubleshooting%20(2).pdf

Practice Problems

- You have been given a method to validate which separates 4-methylaniline and codeine
  - Column – Waters XBridge C18, 4.6 x 100 mm, flow rate 1 mL/min
  - MP = 50:50 MeOH:10 mM sodium bicarbonate, pH 8
- Do you have any concerns do you have about the robustness of the method
  - Why?



Summary

- The preparation of our mobile phase is key for chromatographic success
  - Glassware – detergent?
  - Water and solvent quality
  - Filtration – compatibility and porosity
  - Proper mixing – pH before, measure each component separately
  - Degassing – essential for good performance
- Proper storage is also essential to avoid evaporative loss and bacterial growth
- Know the implications of each of these