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Fundamentals of HPLC

Tom Sirard

Introduction

- PHOTO

- About today's presenter:
  - This webinar will be presented by Tom Sirard. Tom is a Sr. Inside Chemistry Specialist at the Waters Corporate Offices in Milford, MA. He graduated from University of Massachusetts Lowell and has been with Waters for more than 13 years. He has helped hundreds of scientists achieve more successful LC separations through recommending the most appropriate Waters LC Columns for their separations needs.

Outline

- **What is Liquid Chromatography?**
  - Chromatography Technology
  - Three Modes of Liquid Chromatography
  - What is HPLC?
  - Origin of HPLC
- **HPLC System Overview**
  - Review of main components of an HPLC system
- **How an HPLC column works**
  - Sample band vs. analyte band
- **HPLC Detectors**
  - Common types of HPLC detectors
  - How a UV detector works
- **Chromatogram overview**
  - Identification & Quantitation
- **Types of Solvent Runs**
  - Isocratic vs. Gradient
- **Modes of LC Separations**
  - Normal Phase, Reversed-phase, Ion Exchange, Size Exclusion
- **Separation Scale (Analytical, Semi-Prep, etc…)**
- **Particle Shape & Particle Size**
- **Alliance® HPLC System Overview**
- **What is UPLC® Technology?**
- **Educational Books from Waters Corporation**

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What is Liquid Chromatography?

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- **LIQUID CHROMATOGRAPHY**
- **is the**
- **SCIENCE of SEPARATING**
- **the**
- **CHEMICAL COMPOUNDS**
- **that are in the**
- **SAMPLE**

What is Liquid Chromatography?

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- SAMPLE

- **We can then**
- **Identify and Quantitate**
- **What is Present**

Chromatography Technology

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- Several Major Types
  - GC {Gas Chromatography}
  - **LC {Liquid Chromatography}**
    - TLC (**T**hin **L**ayer **C**hromatography
    - Paper Chromatography
    - **HPLC** (**H**igh **P**erformance **L**iquid **C**hromatography)
    - **UPLC®** (**U**ltra **P**erformance **L**iquid **C**hromatography)
    - SPE (**S**olid **P**hase **E**xtraction)
    - Flash Chromatography

Three Modes of Liquid Chromatography

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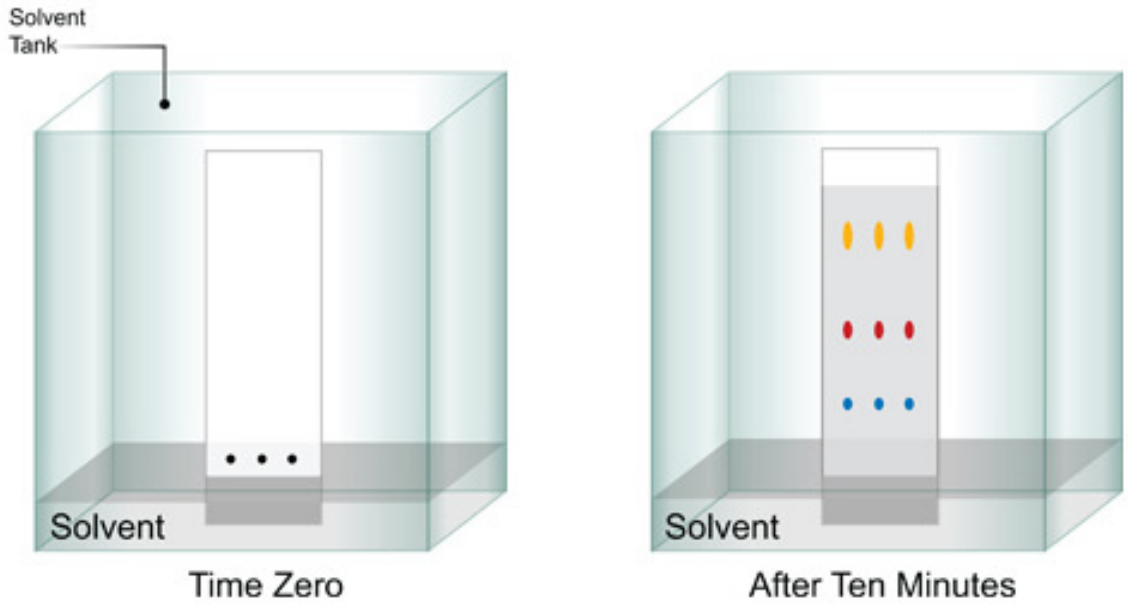
- **Thin Layer Chromatography (TLC)**



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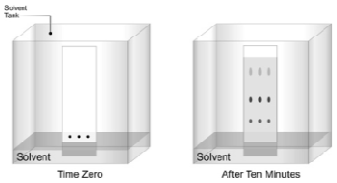
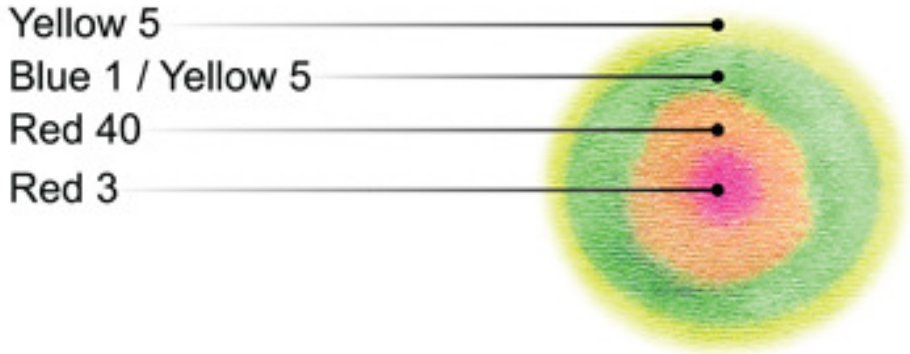


- **Paper Chromatography**



Three Modes of Liquid Chromatography

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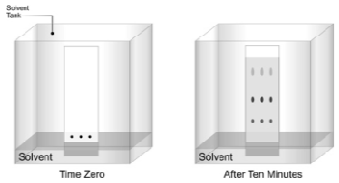
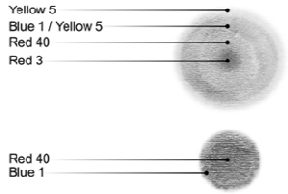
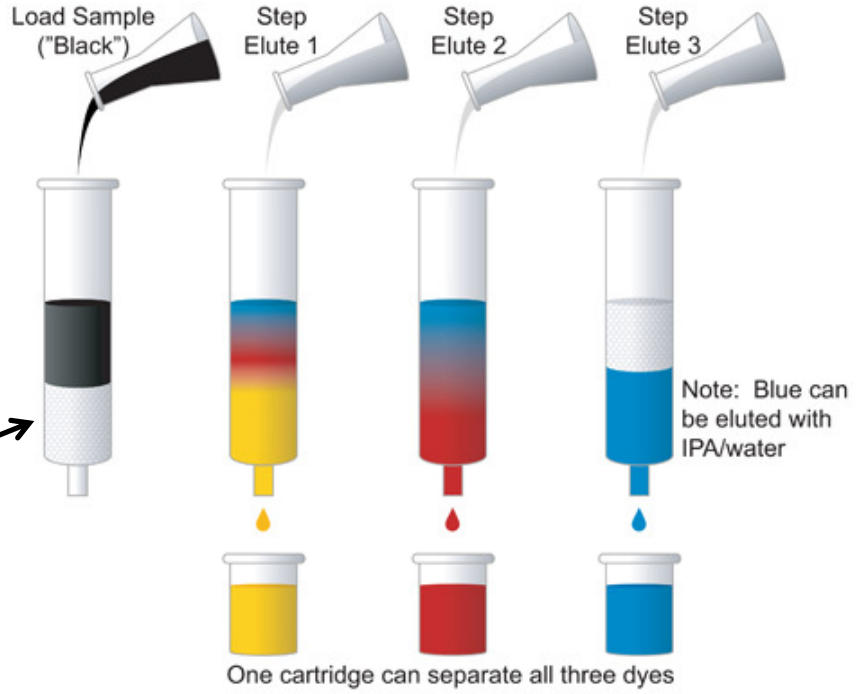


- Paper Chromatography



- **Column Chromatography**
  - **S**olid **P**hase **E**xtraction
  - HPLC
  - UPLC®
  - Flash Chromatography



What is HPLC?

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- **H**igh **P**erformance **L**iquid **C**hromatography (HPLC) is a column Chromatography technique in which:
  - A cartridge or column is packed with a sorbent (stationary phase).
  - A liquid (mobile phase) is passed through the packed column.
  - A dissolved sample (in a liquid) is injected into the flow path of the mobile phase. (this is an "sample band")
  - The sample band separates into individual analyte bands as it passes through the HPLC column.
  - Analytes bands are detected
  - A chromatogram is generated; analyte bands are seen as "peaks"
  - Peaks are quantitated

Origin of Liquid Chromatography: Dr. Mikhail Tswett's Experiment (1903)

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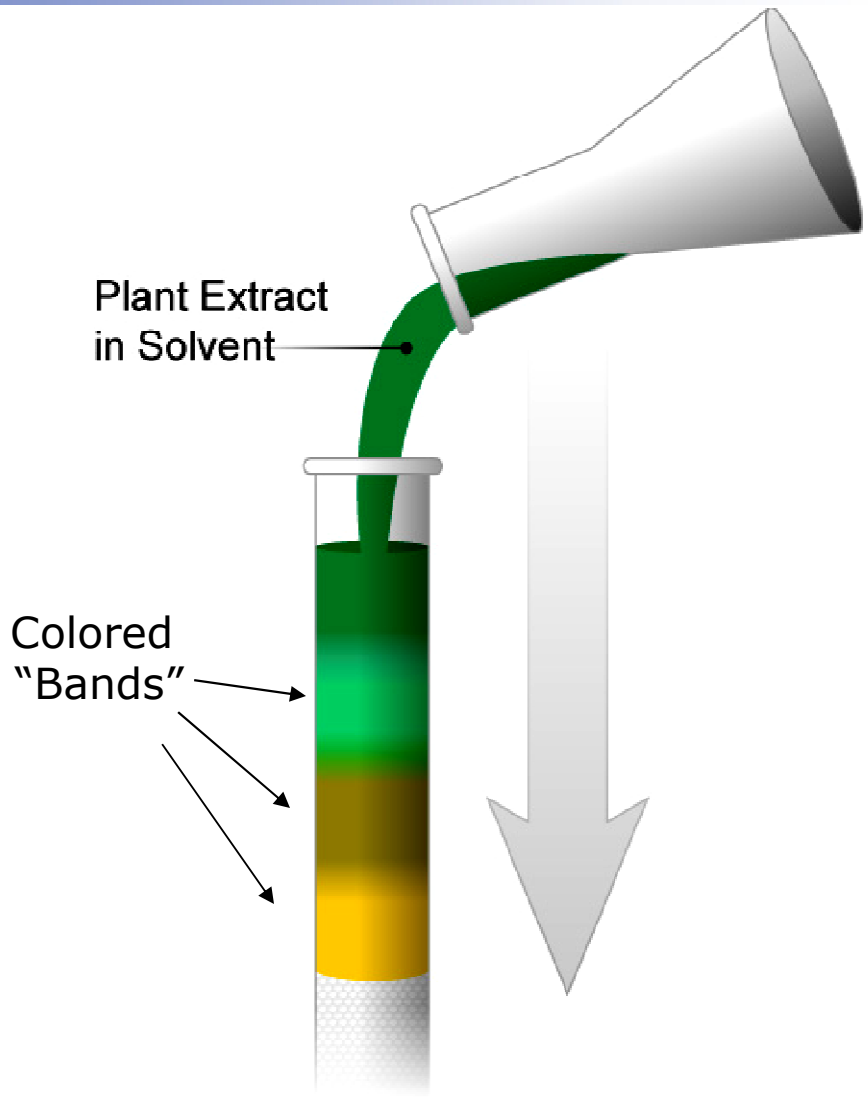
- Tall glass open column filled with sand-like particles
- Ground-up plant extract
- Poured into the column and saw colored "bands" develop as the extract percolated down thru the column
- Different compounds had separated



Greek

Chroma -- color
Graphy -- writing/study of

Note: "Tswett" in Russian means Color

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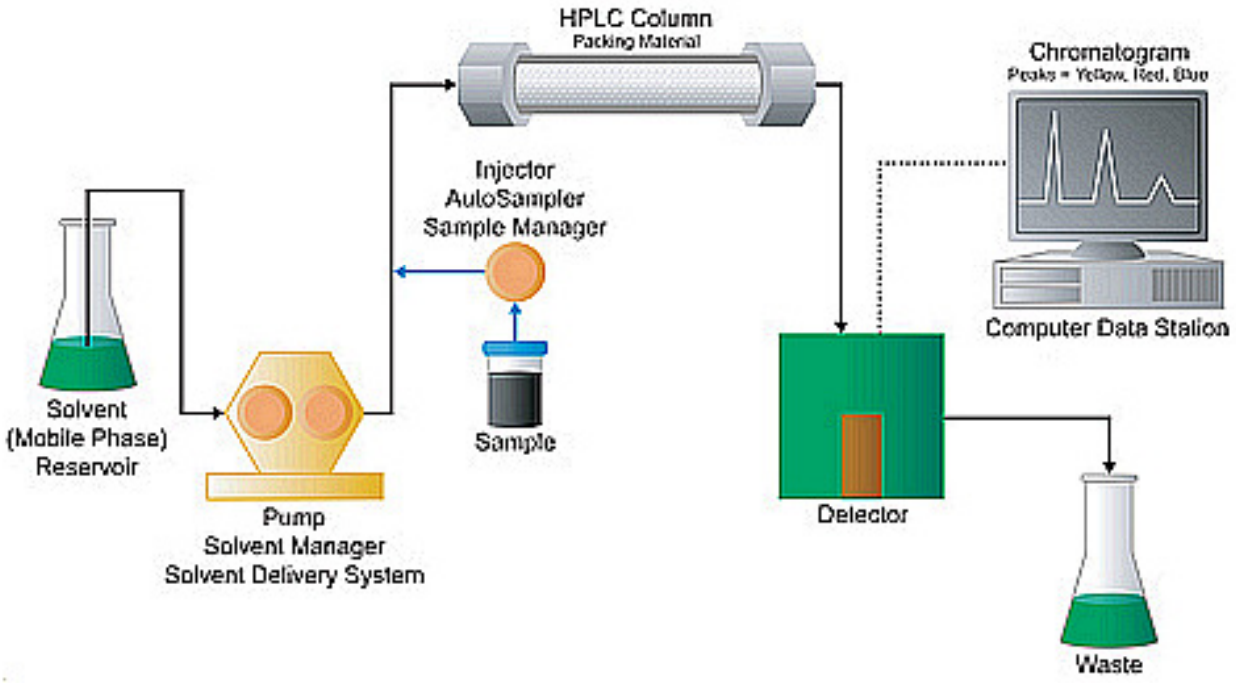
HPLC System Diagram



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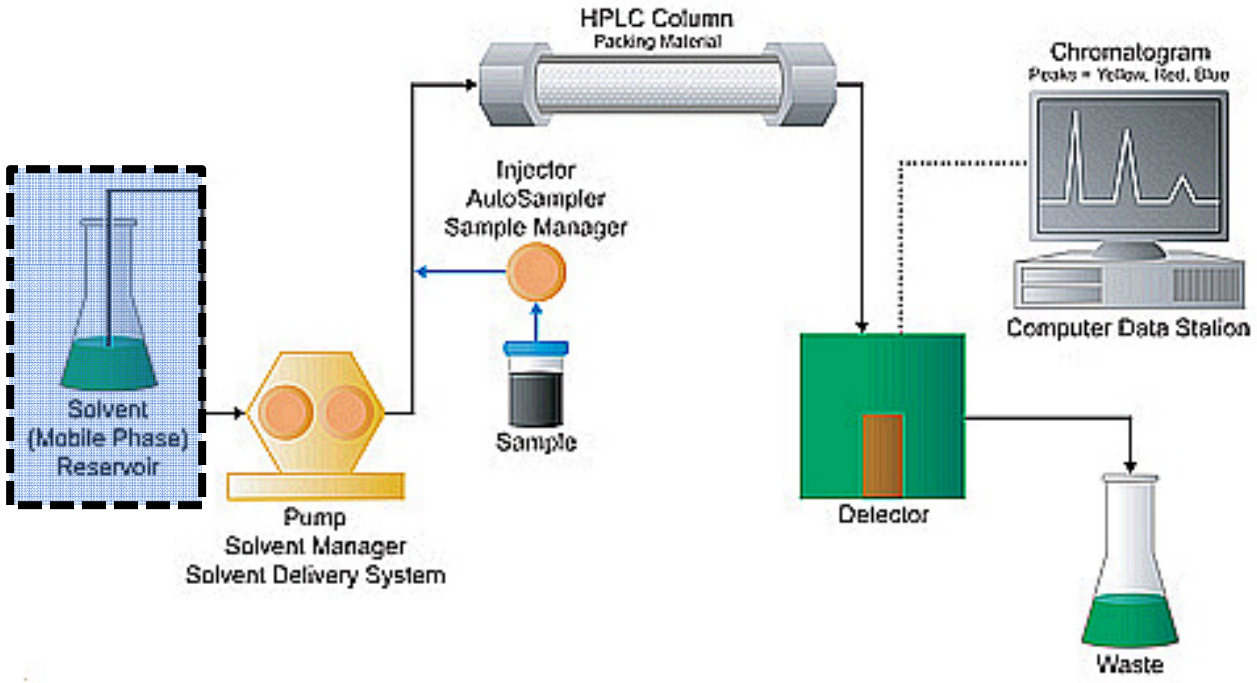

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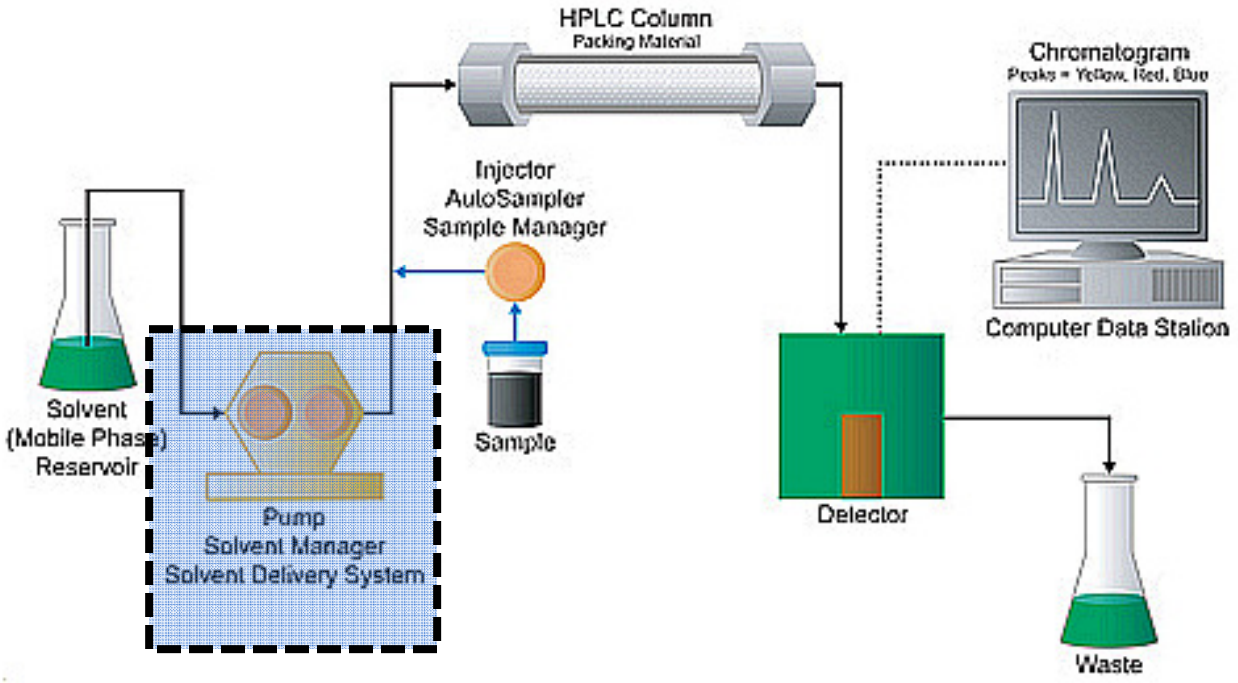

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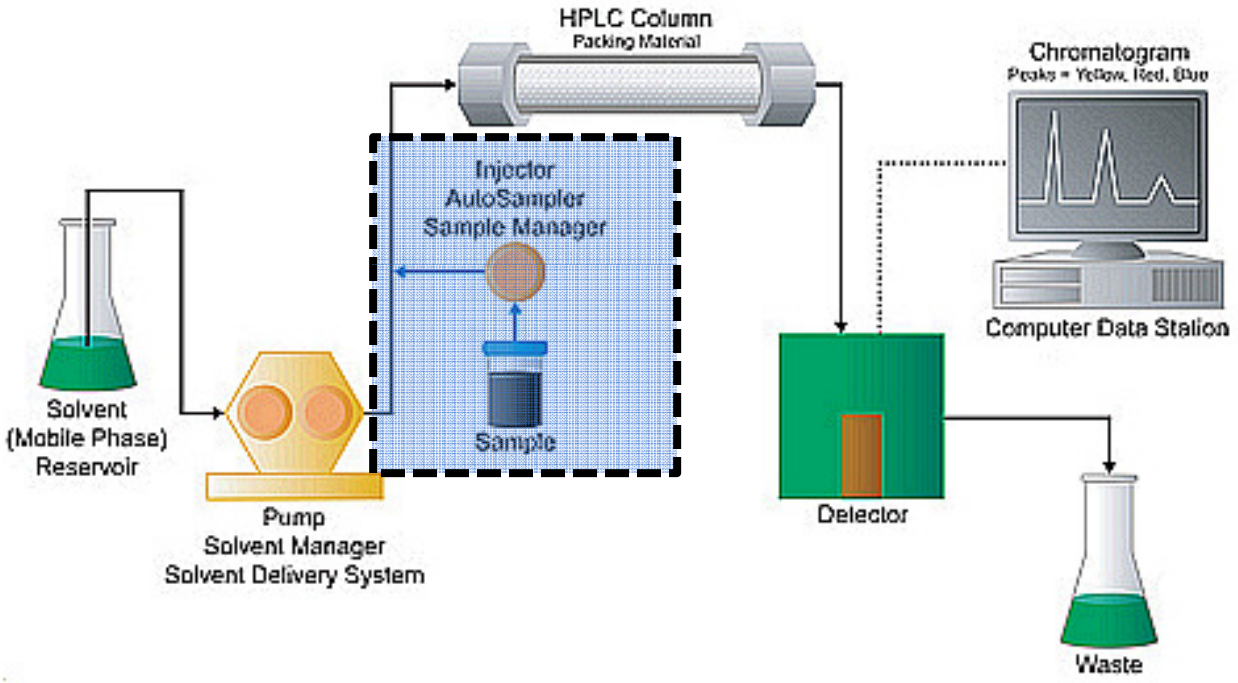

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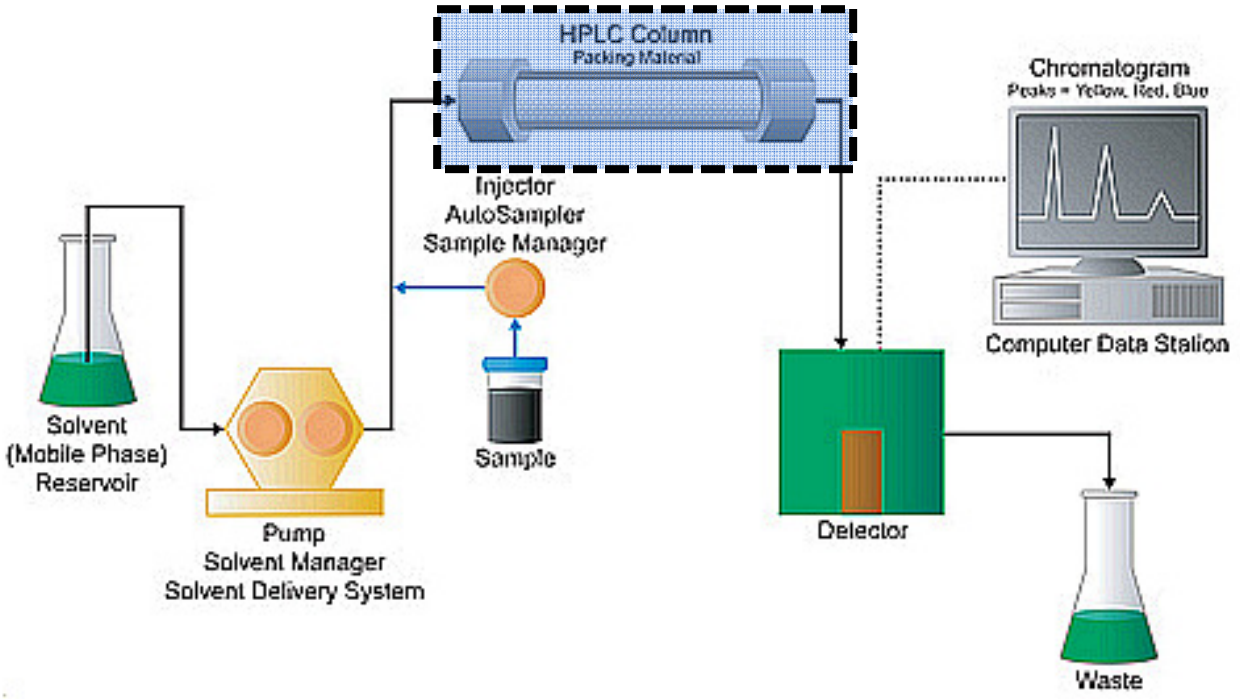

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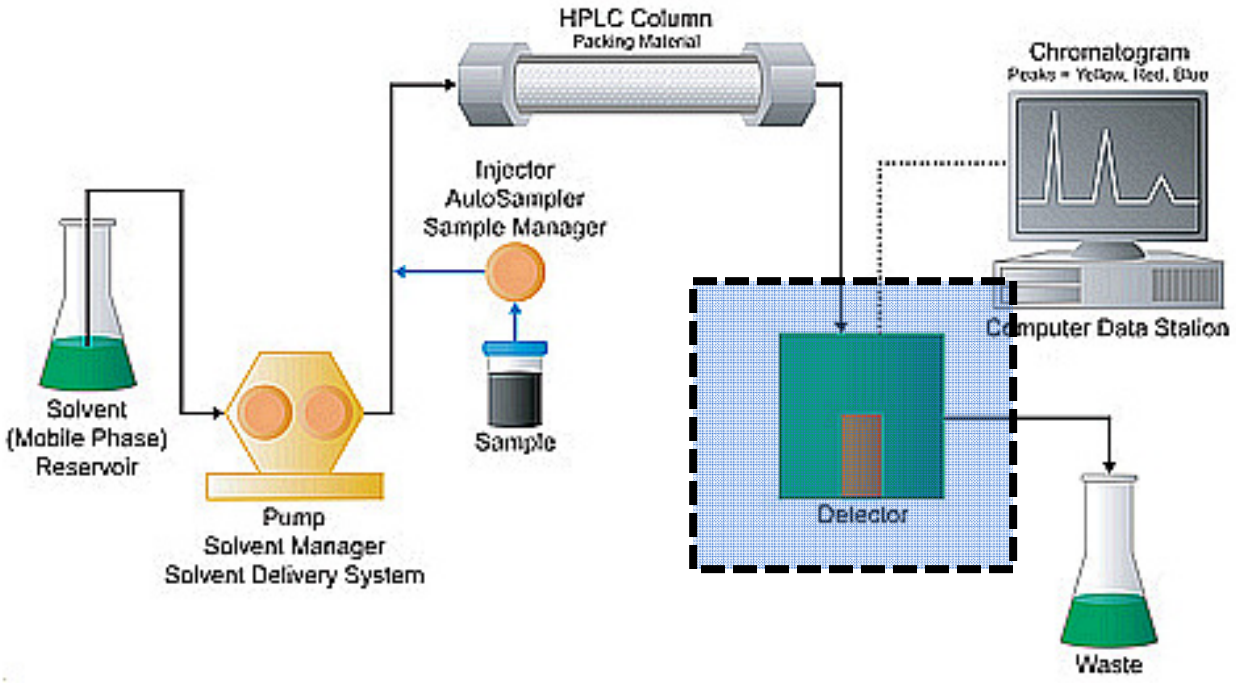

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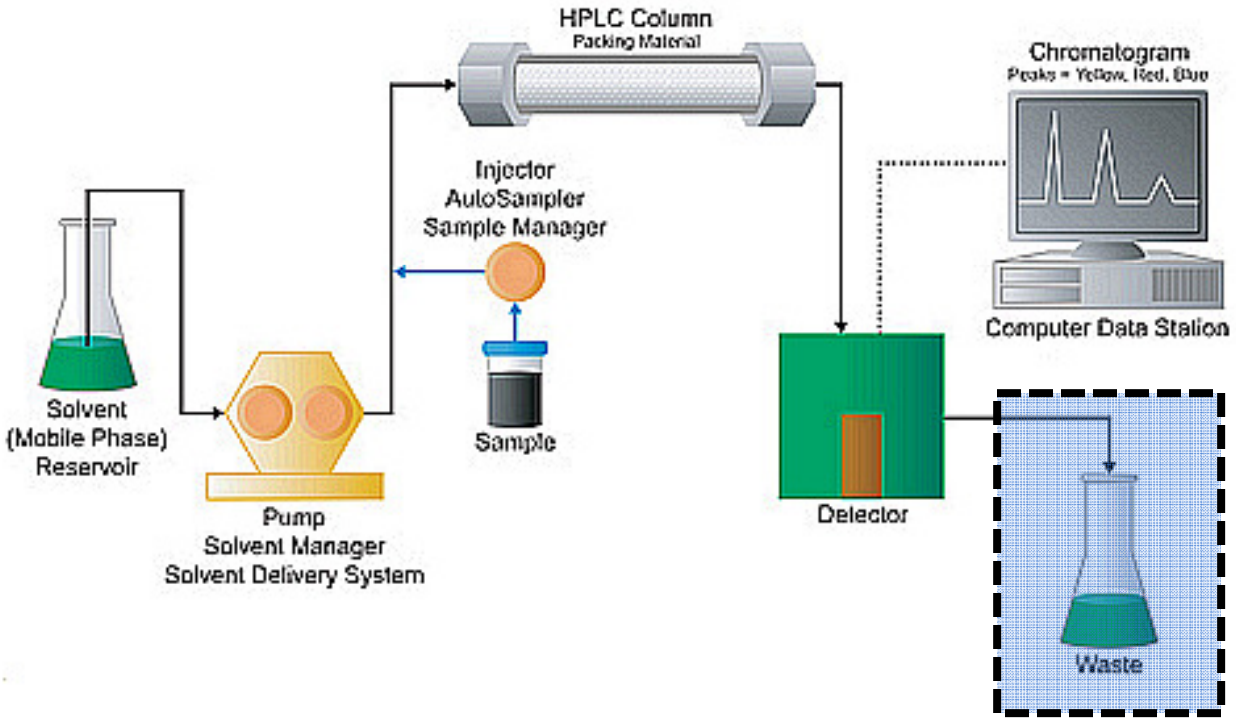

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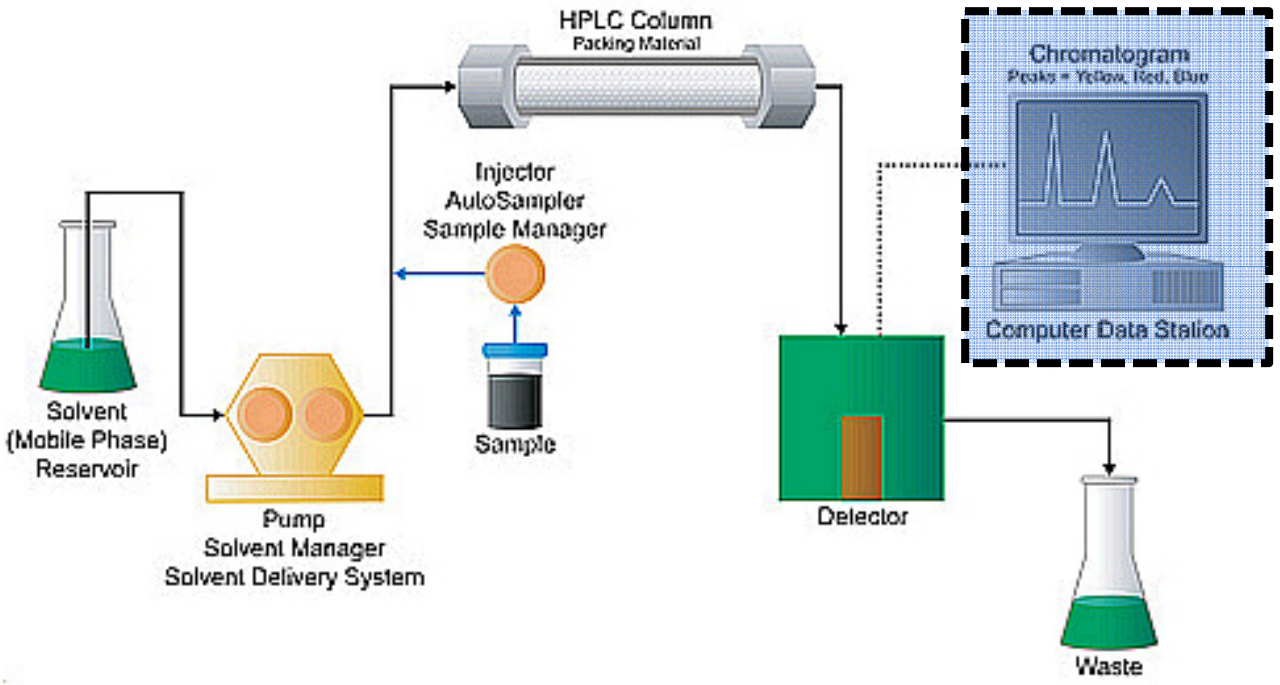

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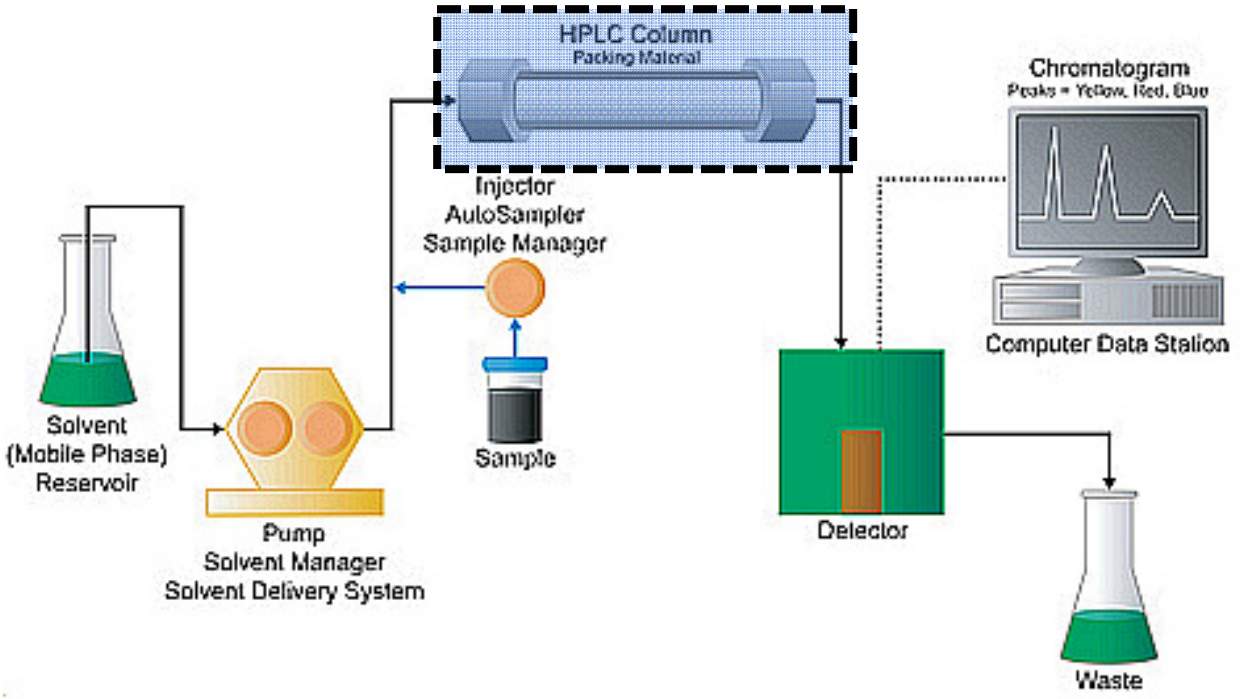

Understanding How a Chromatographic Column Works – *"BANDS"*

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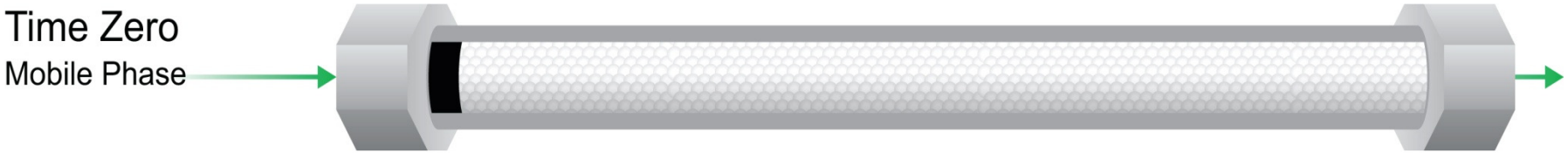
We create a separation by changing the ***relative speed*** of *each* analyte band
(competition between the mobile phase and stationary phase)

Injected Sample Band (Appears "Black") (Blue, Red, Yellow)



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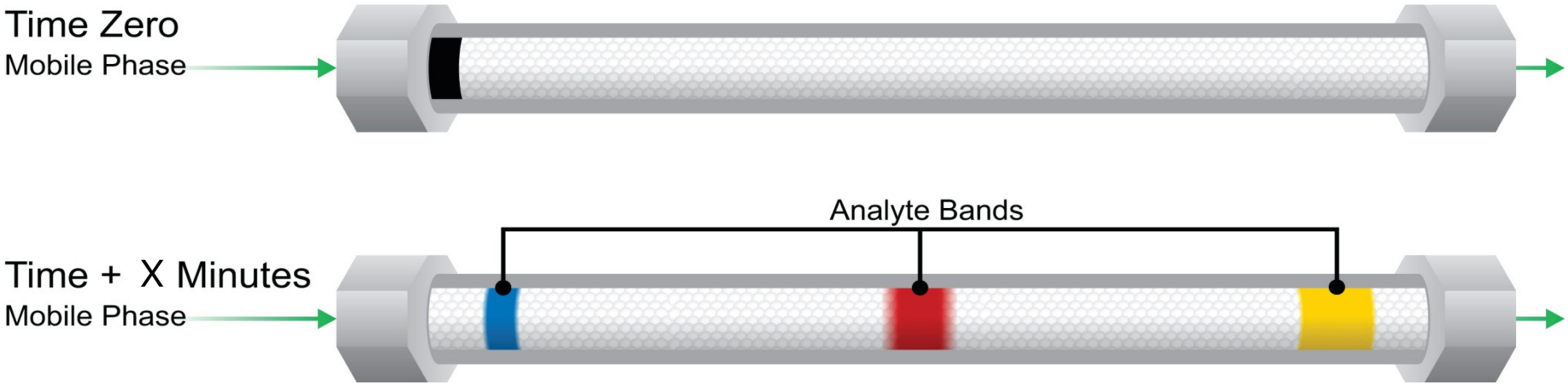

Yellow is the earliest eluting analyte "band" (it will be broader in the column), but moving fastest – it "likes" the mobile phase

Blue is well retained, it will be in a more focused, narrower band, near the inlet and move the slowest in the column – it "likes" the particles

A Look Inside a Column

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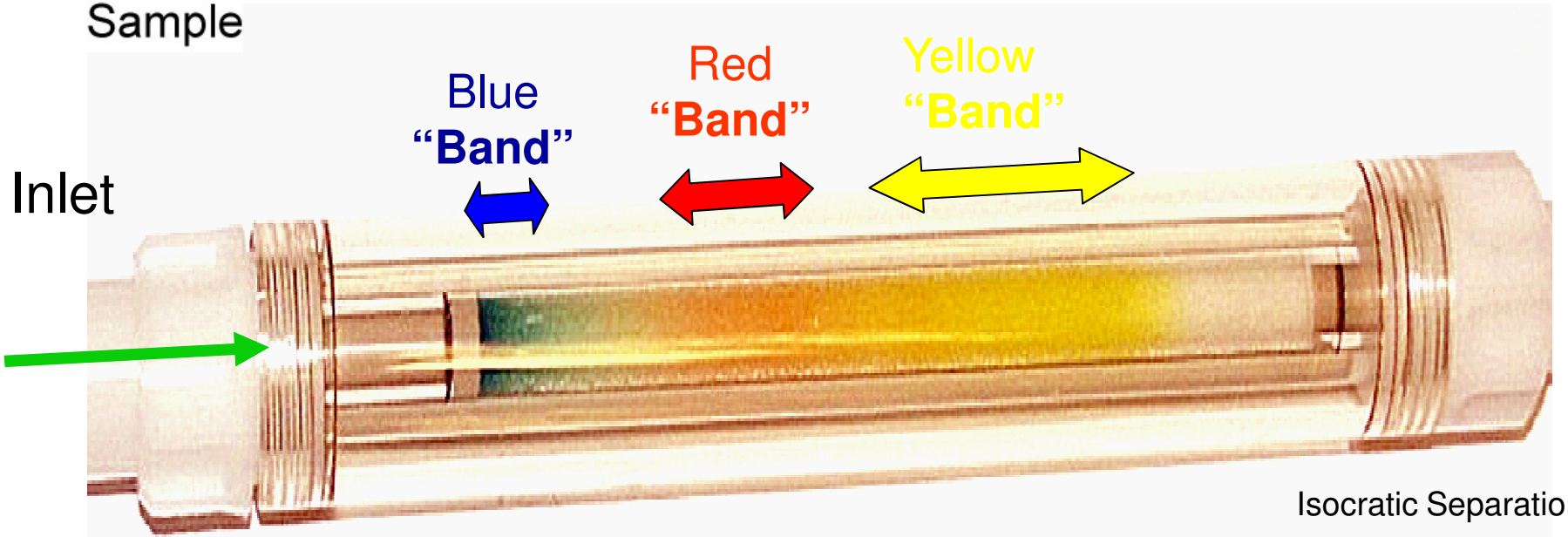
Sample

Sample: Black "Ink-like" solution made from a mixture of Blue, Red and Yellow Dyes



Isocratic Separation

The Separation is actually CREATED in the LC Column

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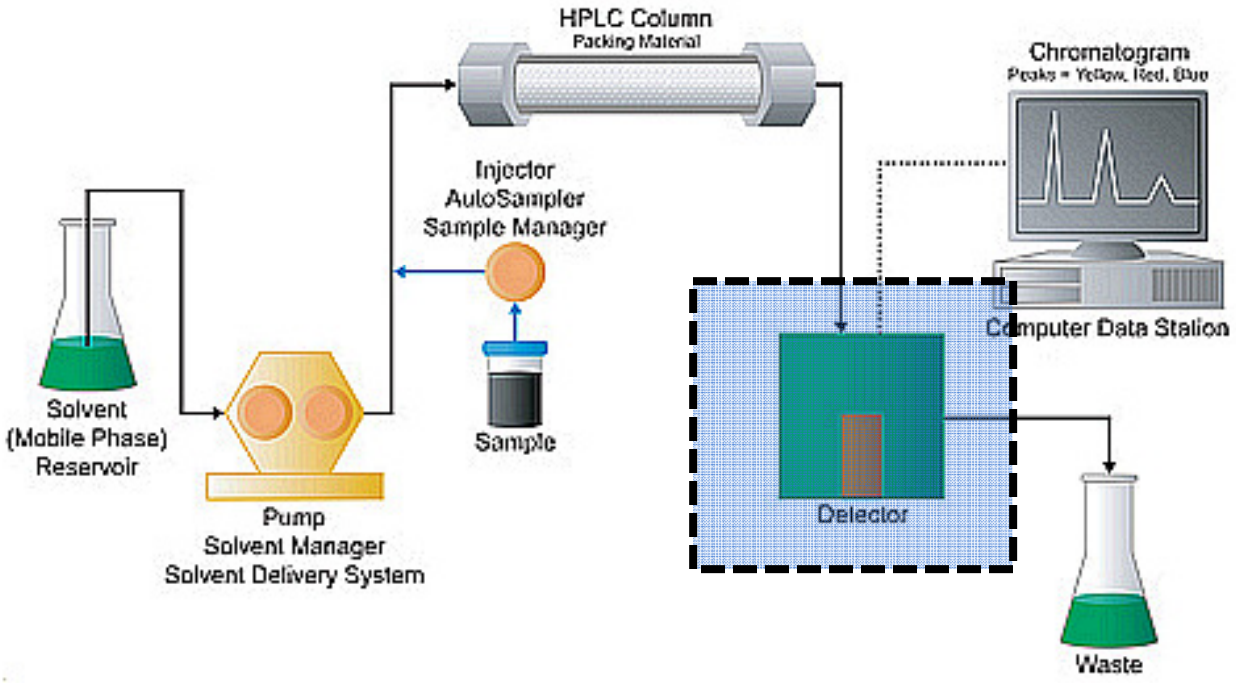

Detectors

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Different compounds require different detectors to be "seen"

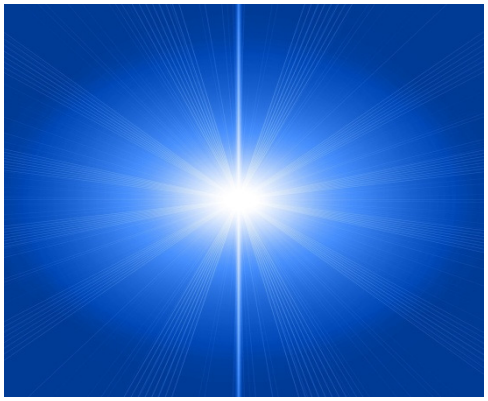
- UV
- Fluorescence
- Evaporative Light Scattering
- Refractive Index
- Electrochemical

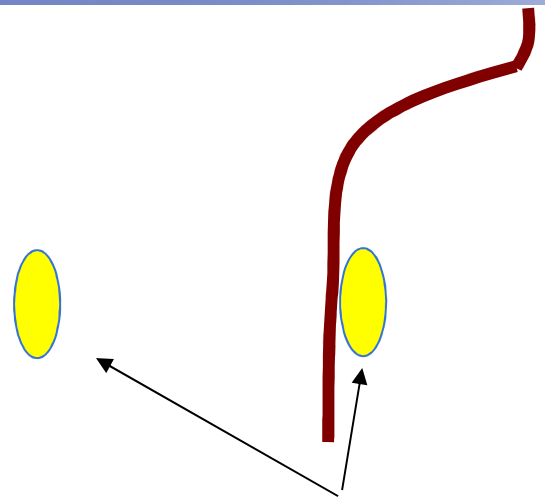
Mass Spectrometer

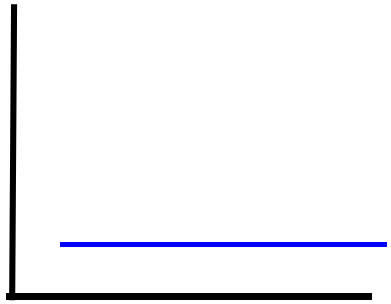
[actually tells you something about each compound, to better identify them]

LC/MS

Concept of a UV Absorbance Detector







Light Sensors measure the SAME amount of Light – NO SIGNAL

Concept of a UV Absorbance Detector

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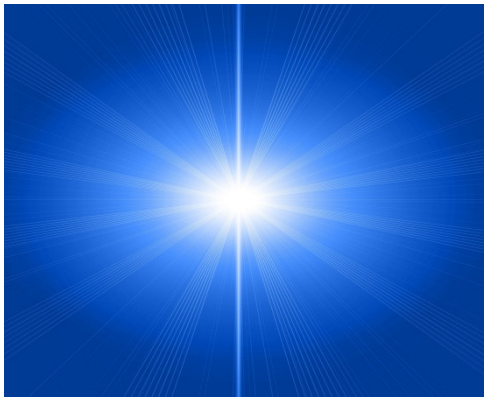

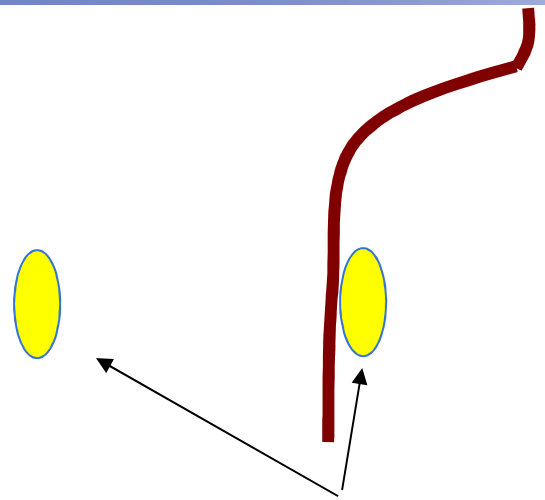

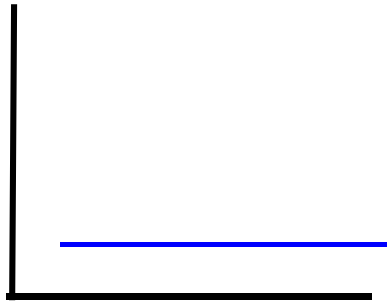

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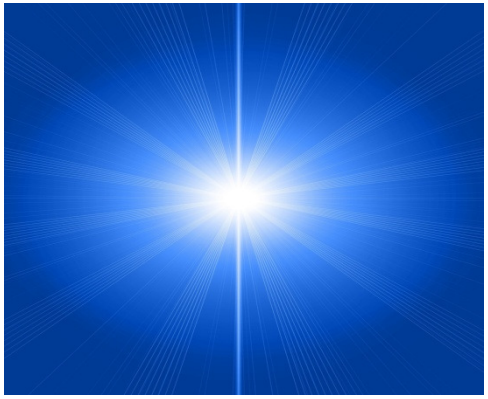

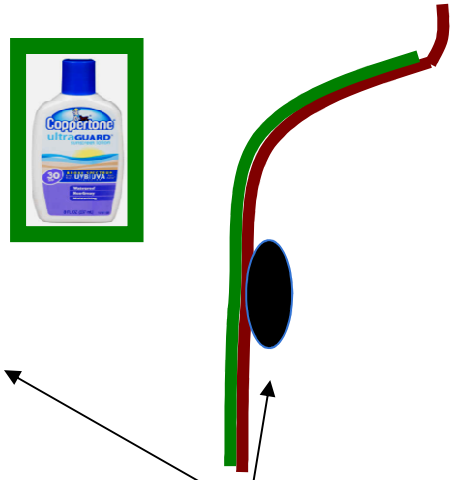

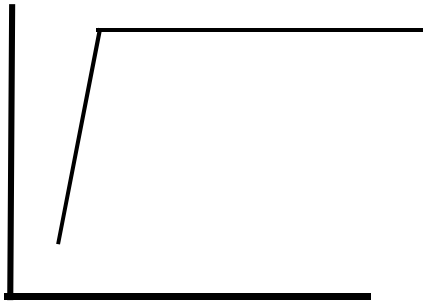

Light Sensors measure DIFFERENT amounts of Light – LARGE SIGNAL

SunScreen ABSORBS UV Light

Concept of a UV Absorbance Detector

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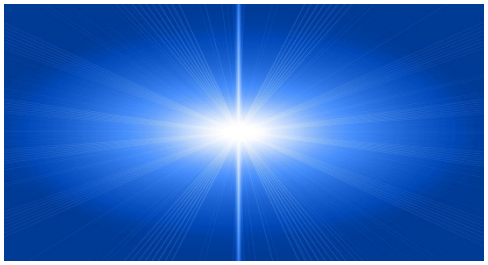

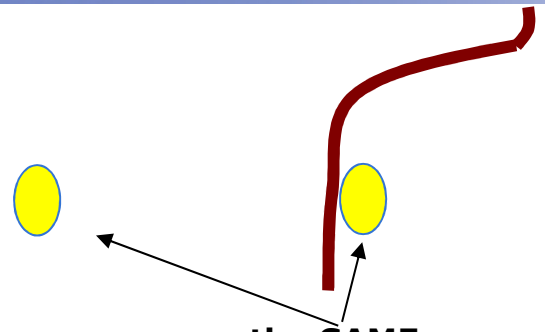

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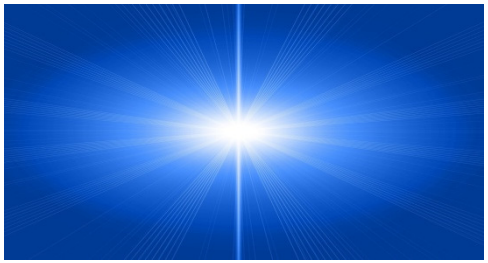
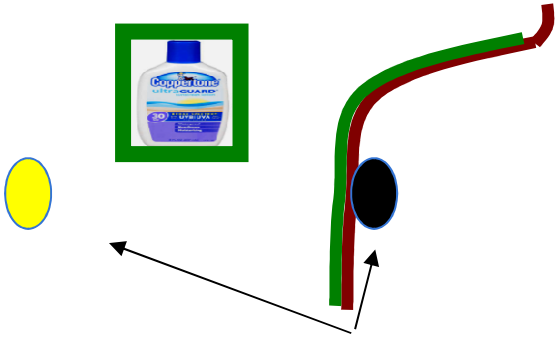
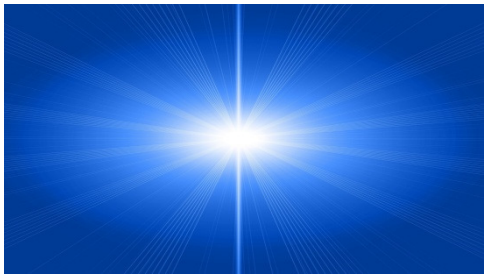
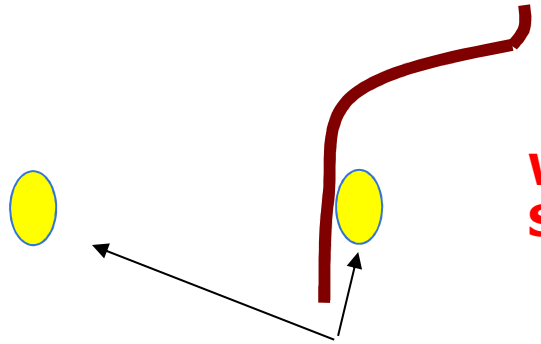
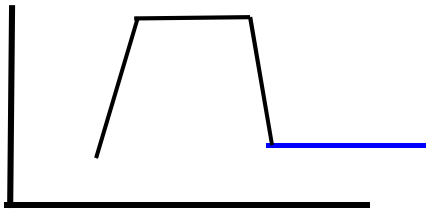








Wash Off SunScreen



Light Sensors measure the SAME amount of Light – NO SIGNAL

UV Detector
Ultra Violet Light

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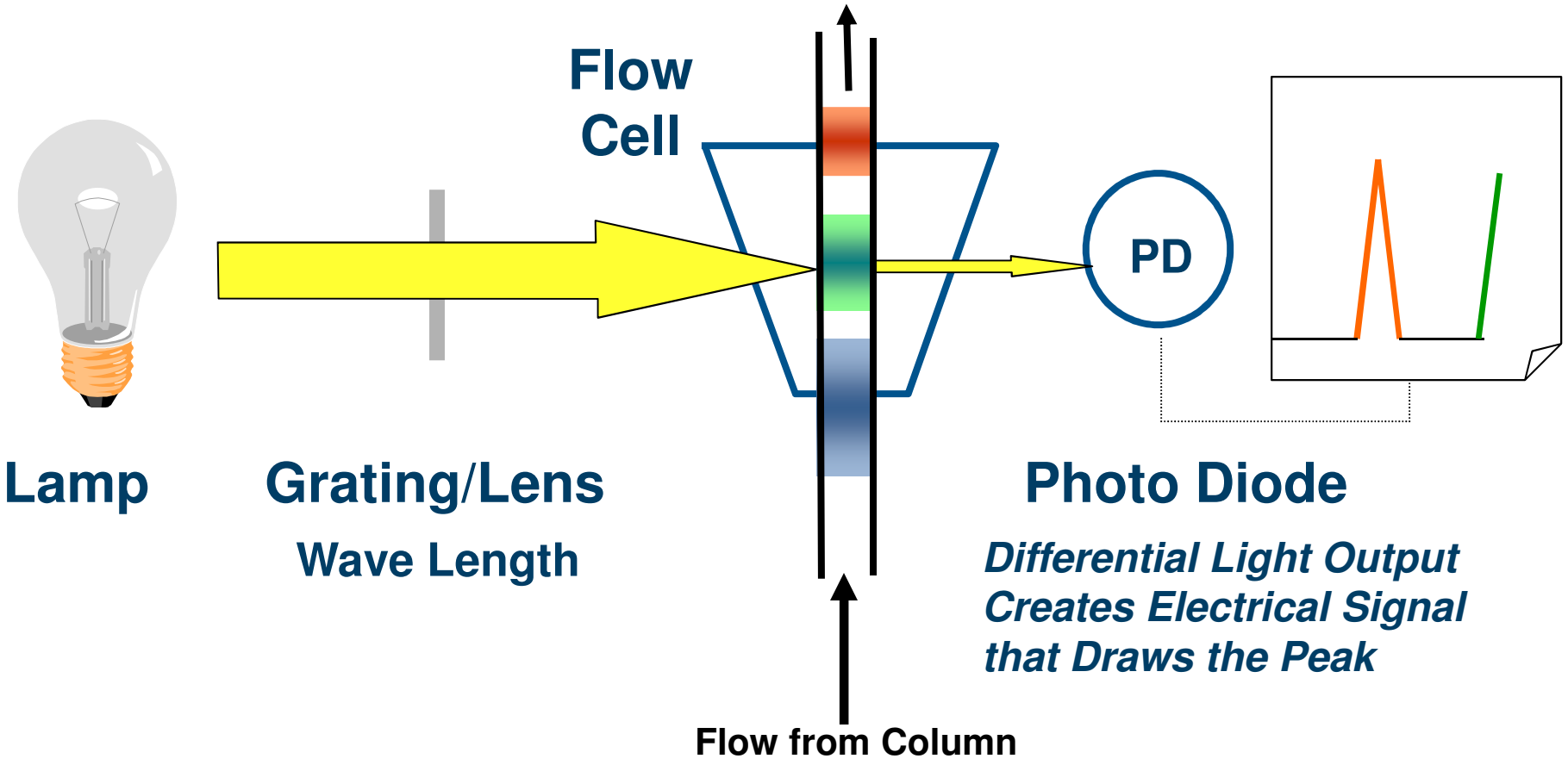
Will only work for compounds that **absorb** UV Light



Example: SunScreen

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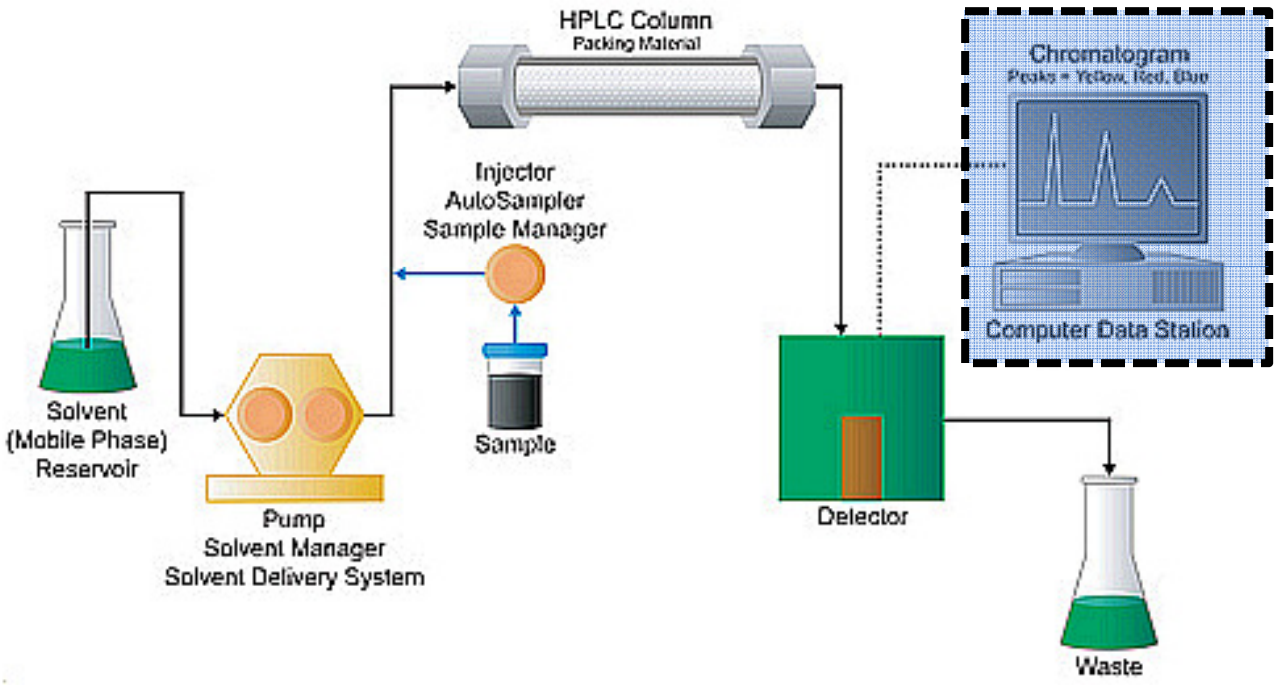
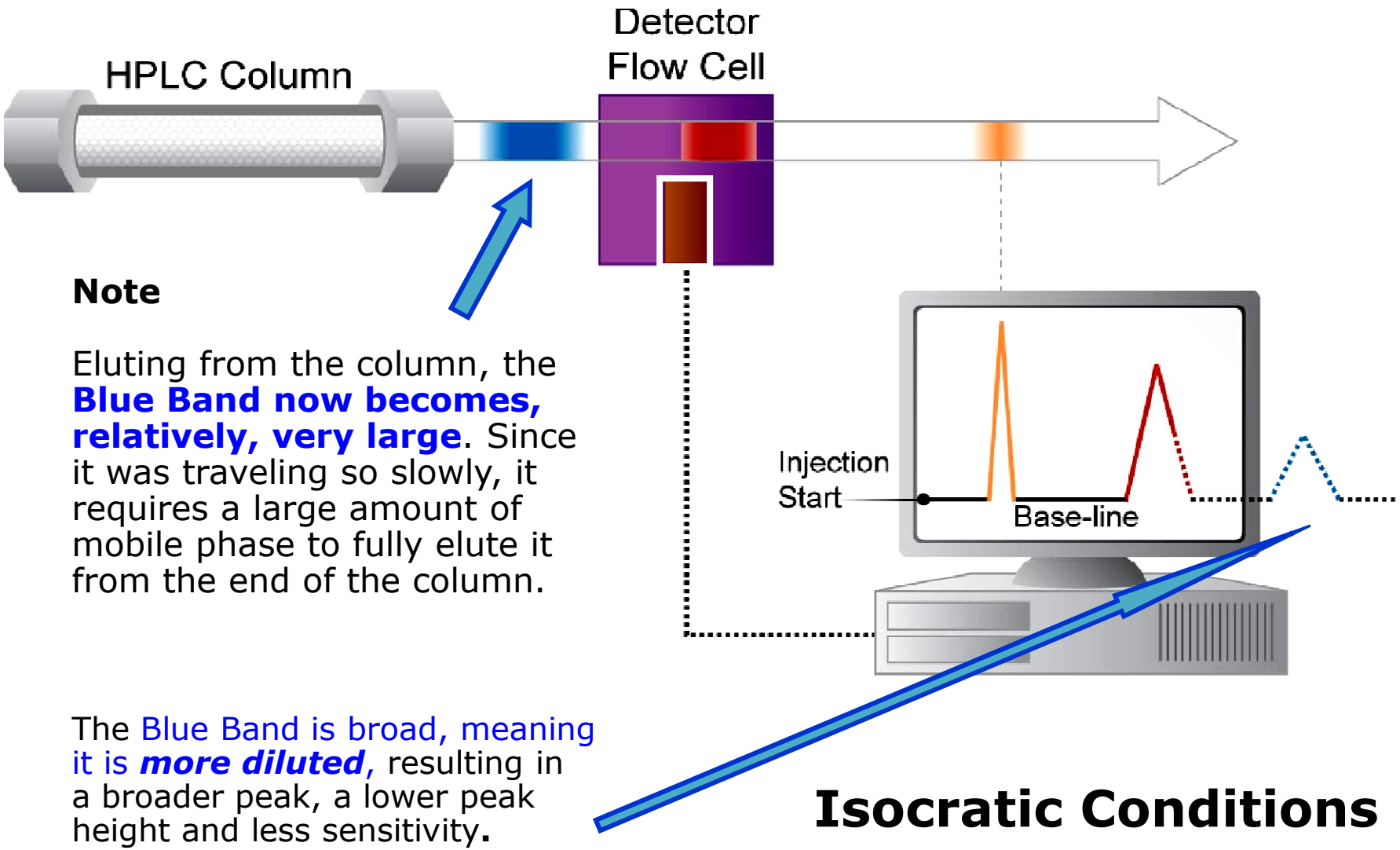
HPLC System Diagram



How are Peaks Created



Note

Eluting from the column, the **Blue Band now becomes, relatively, very large**. Since it was traveling so slowly, it requires a large amount of mobile phase to fully elute it from the end of the column.

The Blue Band is broad, meaning it is ***more diluted***, resulting in a broader peak, a lower peak height and less sensitivity.

Isocratic Conditions

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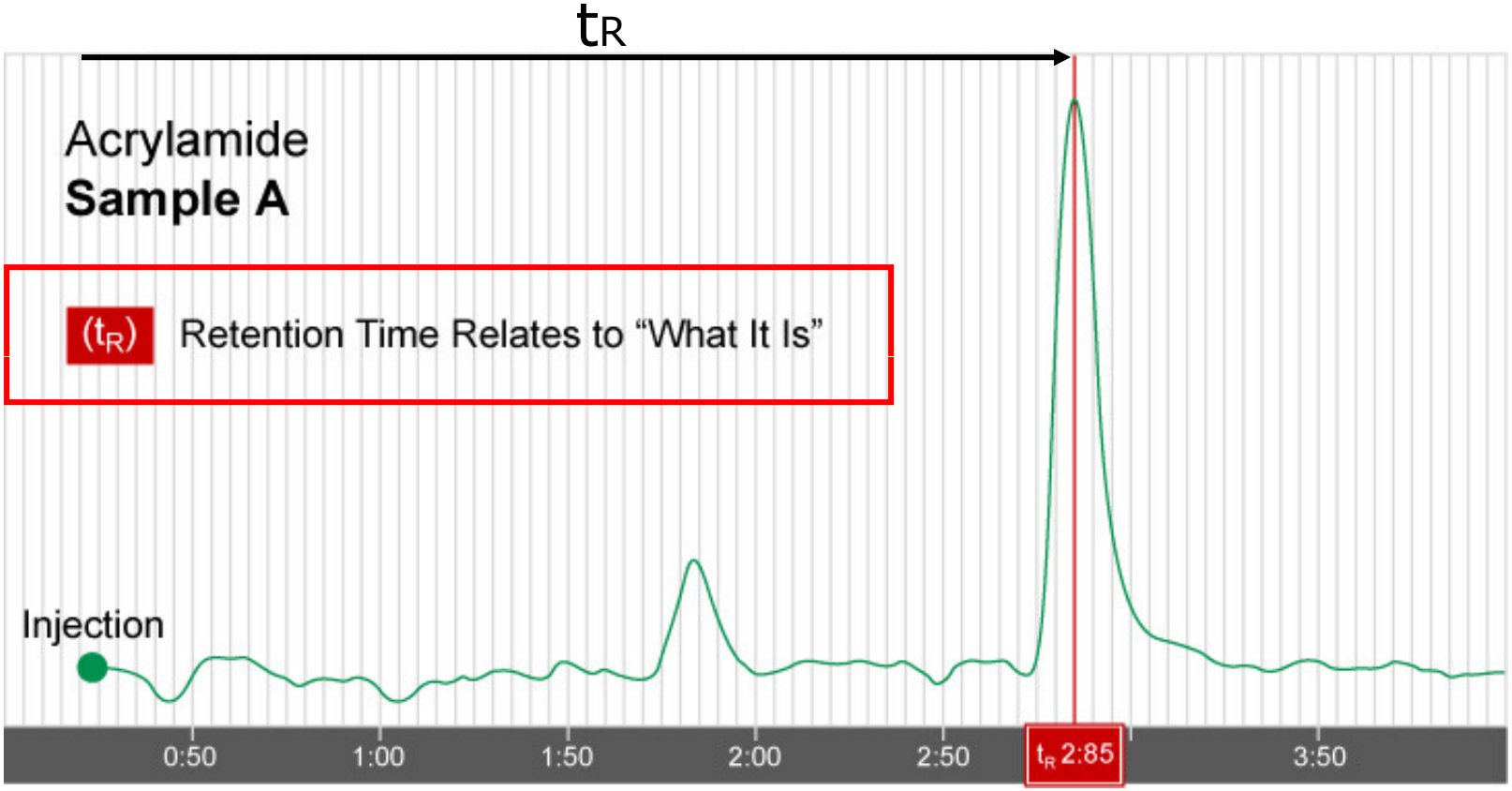
Compound Identification Based on Retention Time



For a given mobile phase, at a given flow rate with a given column, a ***known pure standard of acrylamide*** elutes at 2.85 minutes.
Whenever a real sample is injected that contains acrylamide, you will see a peak at 2.85 minutes.

Identification and Quantitation

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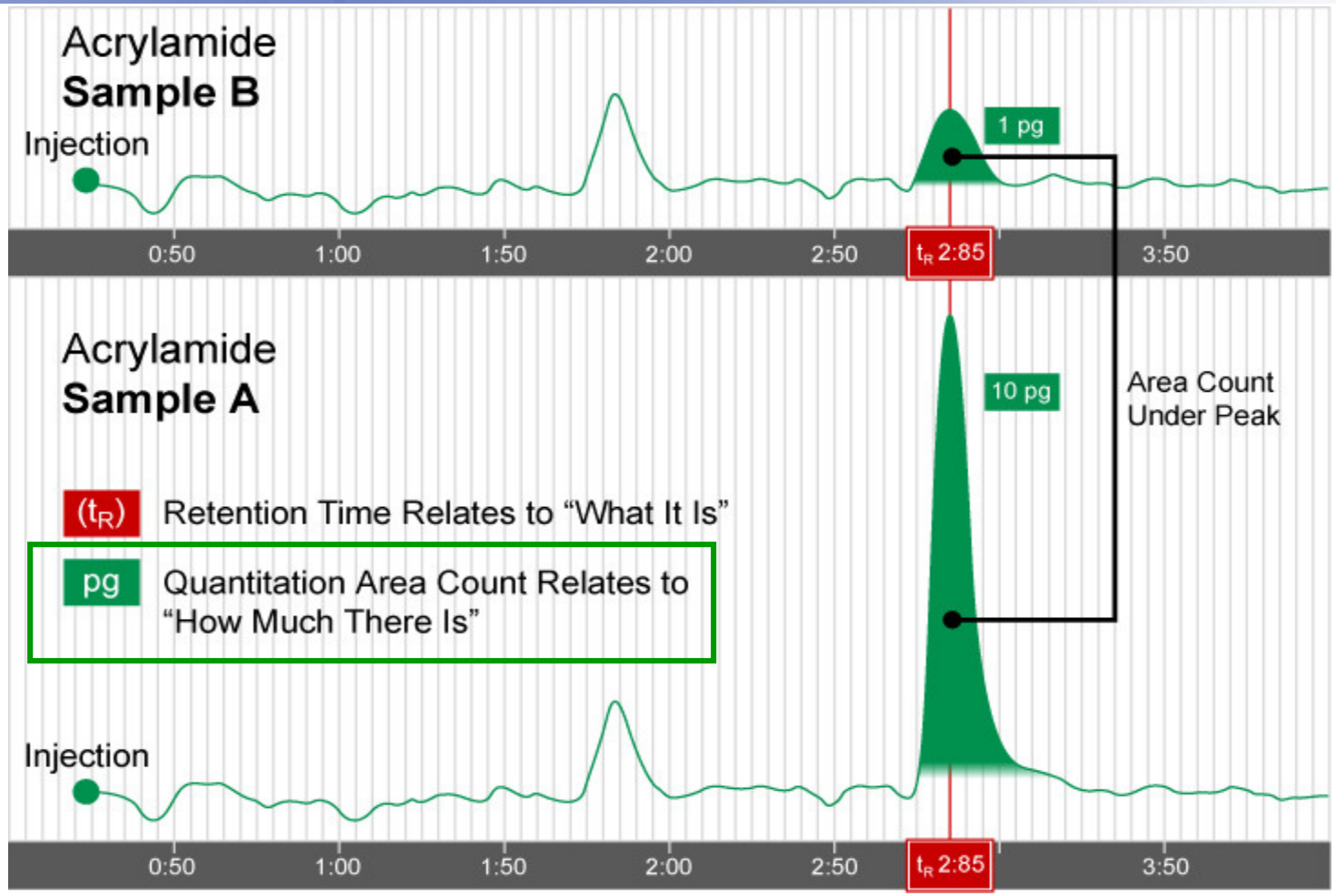

How much is present is measured by the AREA under the peak, which is related to how much was there. Both samples contain acrylamide, however, Sample B has only 1/10 the concentration

Identification and Quantitation

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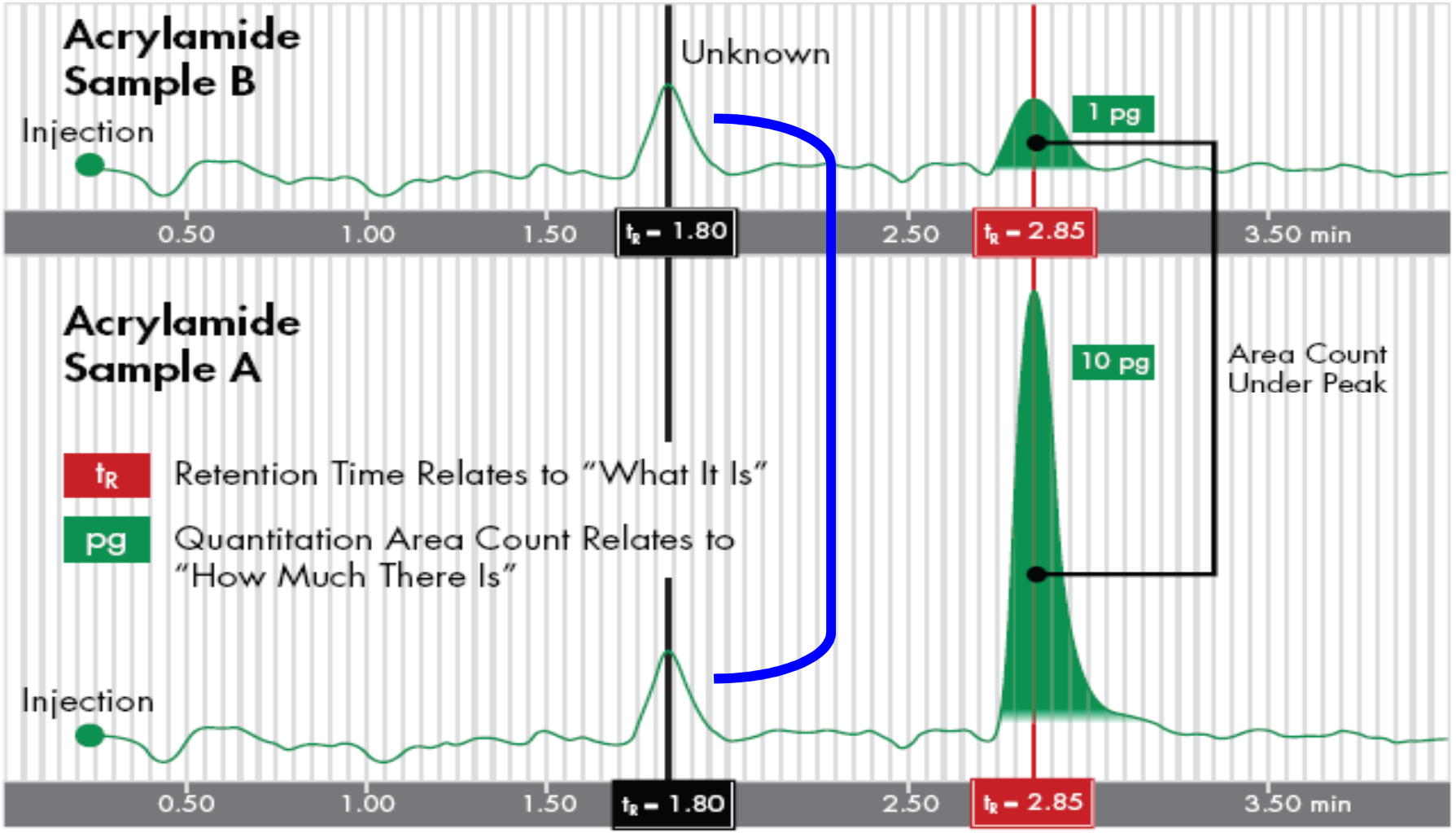

Both samples have ~ SAME amount of this unknown compound

Outline

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- **Modes of LC Separations**
  - Reversed-phase, Normal Phase, Ion Exchange, Size Exclusion
- **Separation Scale (Analytical, Semi-Prep, etc…)**
- **Particle Size**
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Basic Types of LC Solvent Runs

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ISOCRATIC

- **ISO ==> SAME**
- **Solvent Composition Stays the Same for the Entire Run**
  {60:40 Alcohol:Water}

GRADIENT

- **Solvent Composition Changes Throughout the Run**

- **Gradually Changed or Step Changes**

Basic Types of LC Solvent Runs
Isocratic

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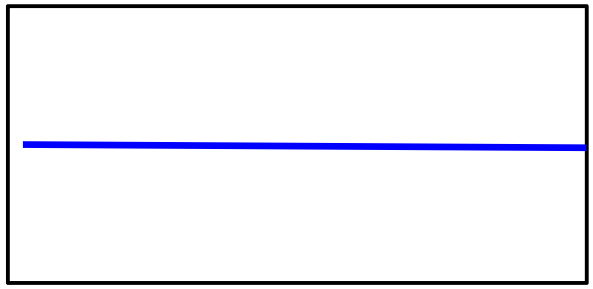
Solvent Strength of Mobile Phase during a Run

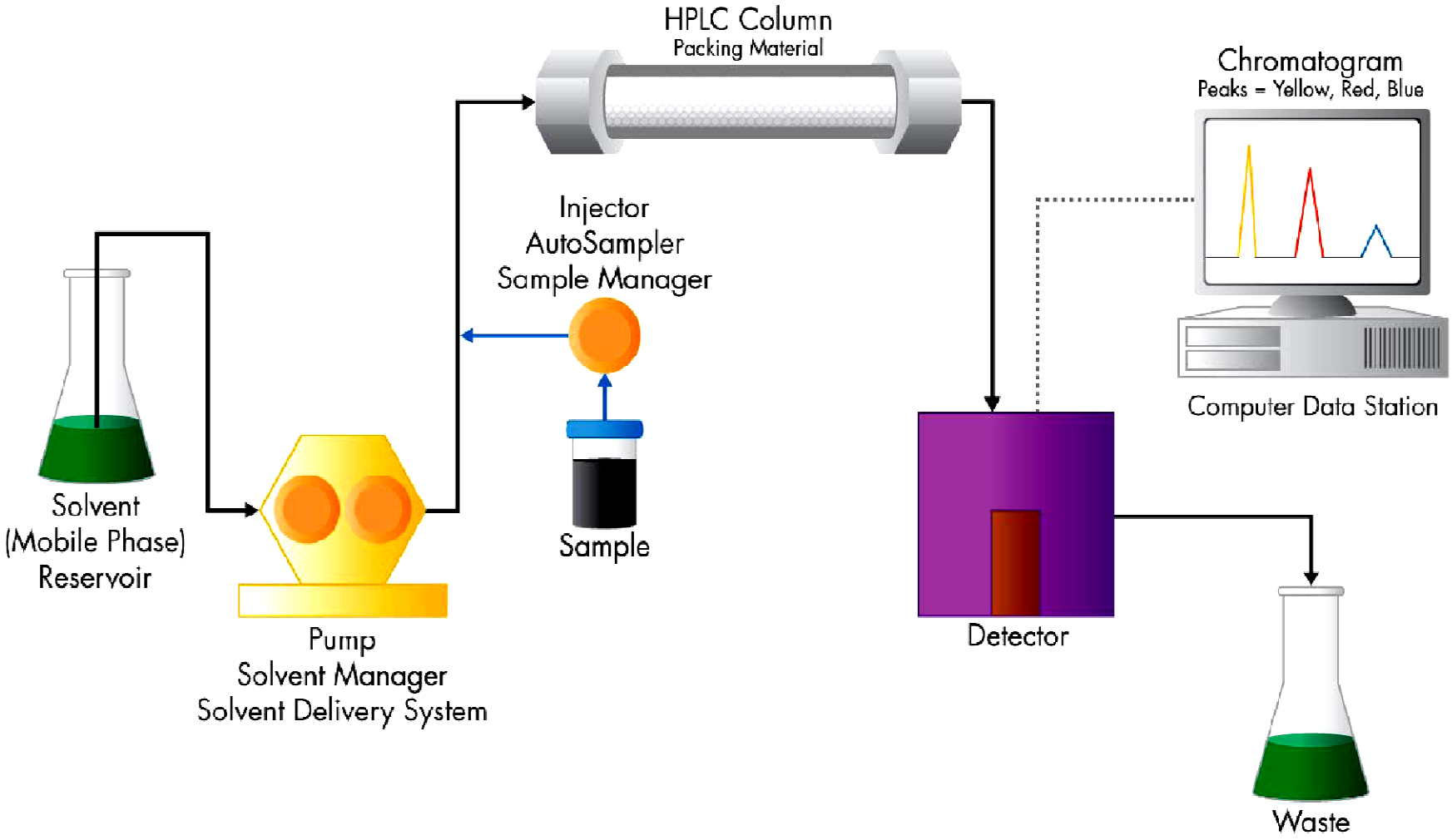
Isocratic



Same

"Iso" = Same

Isocratic LC System



The mobile phase composition **stays the same** throughout the separation

Basic Types of LC Solvent Runs
Gradient

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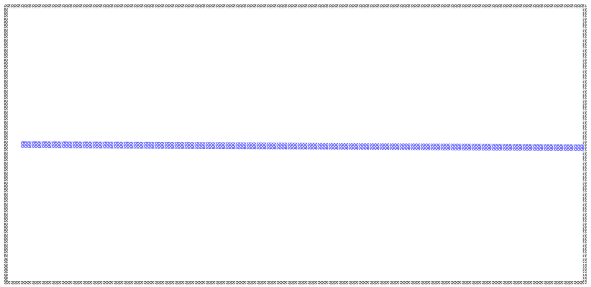
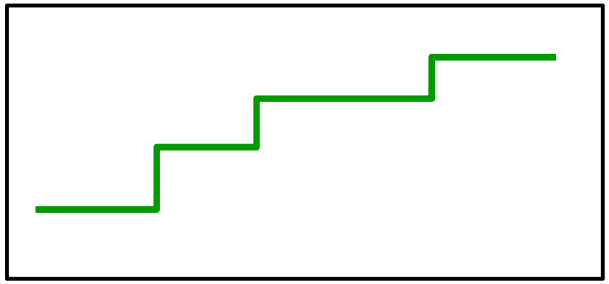
Solvent Strength of Mobile Phase during a Run

Isocratic



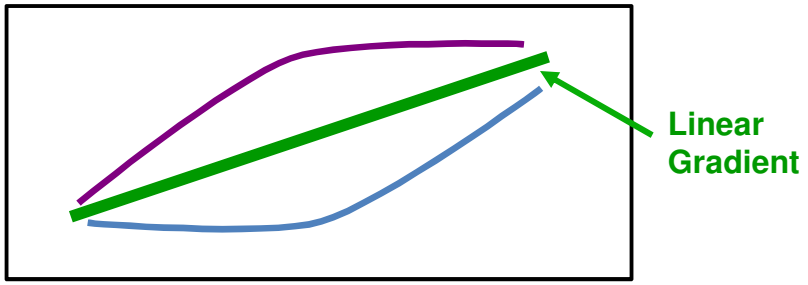
Same

Step-Gradient



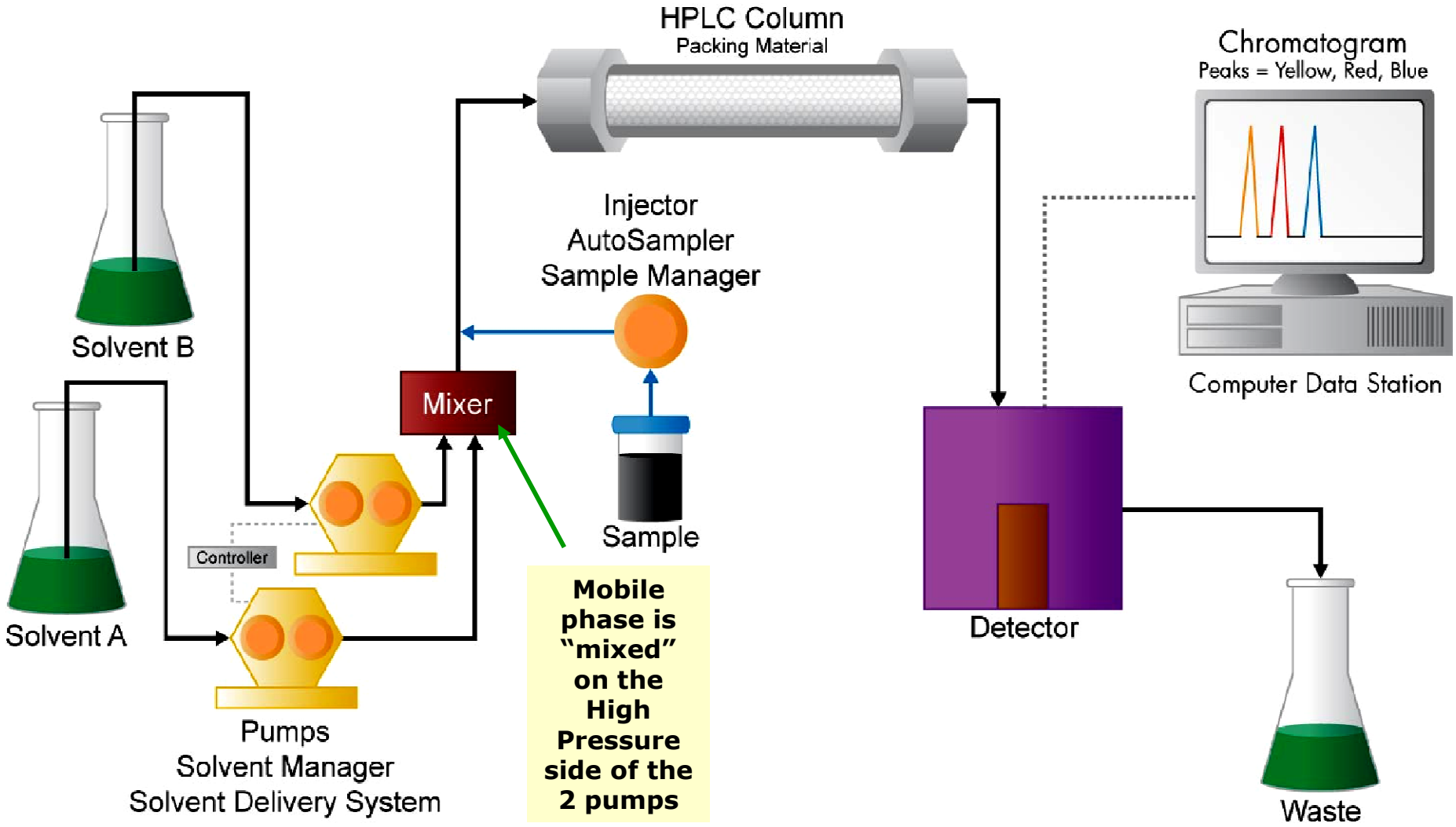
Increasing Strength

Gradient Curves



Increasing Strength

"Multi-Pump" Gradient System High Pressure



The mobile phase composition **changes (becomes stronger) during** the separation

"Single Pump" Gradient System Low Pressure

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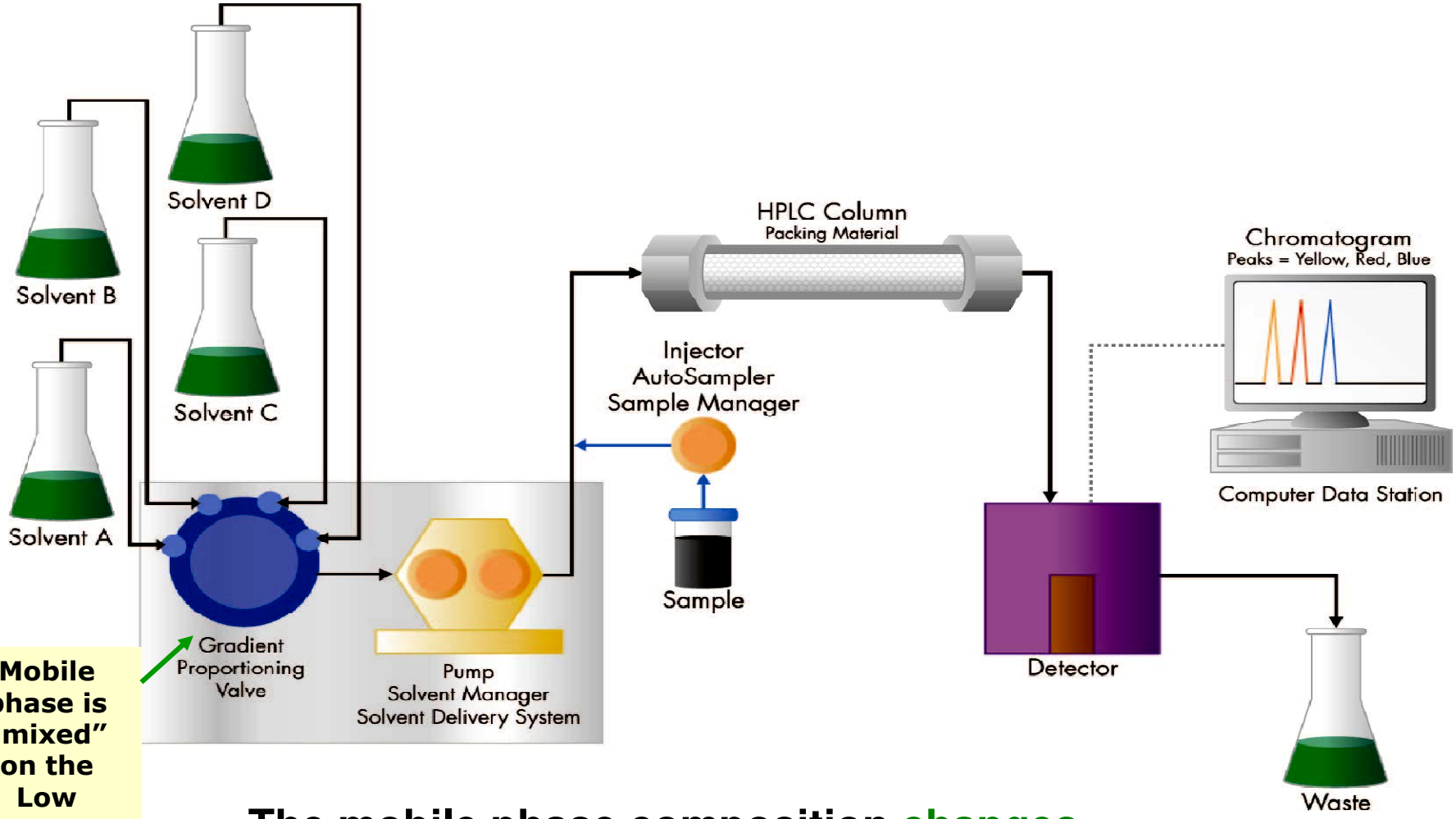

Mobile phase is "mixed" on the Low Pressure side of the pump

The mobile phase composition changes (becomes stronger) during the separation

High Resolution Peptide Mapping: Gradient

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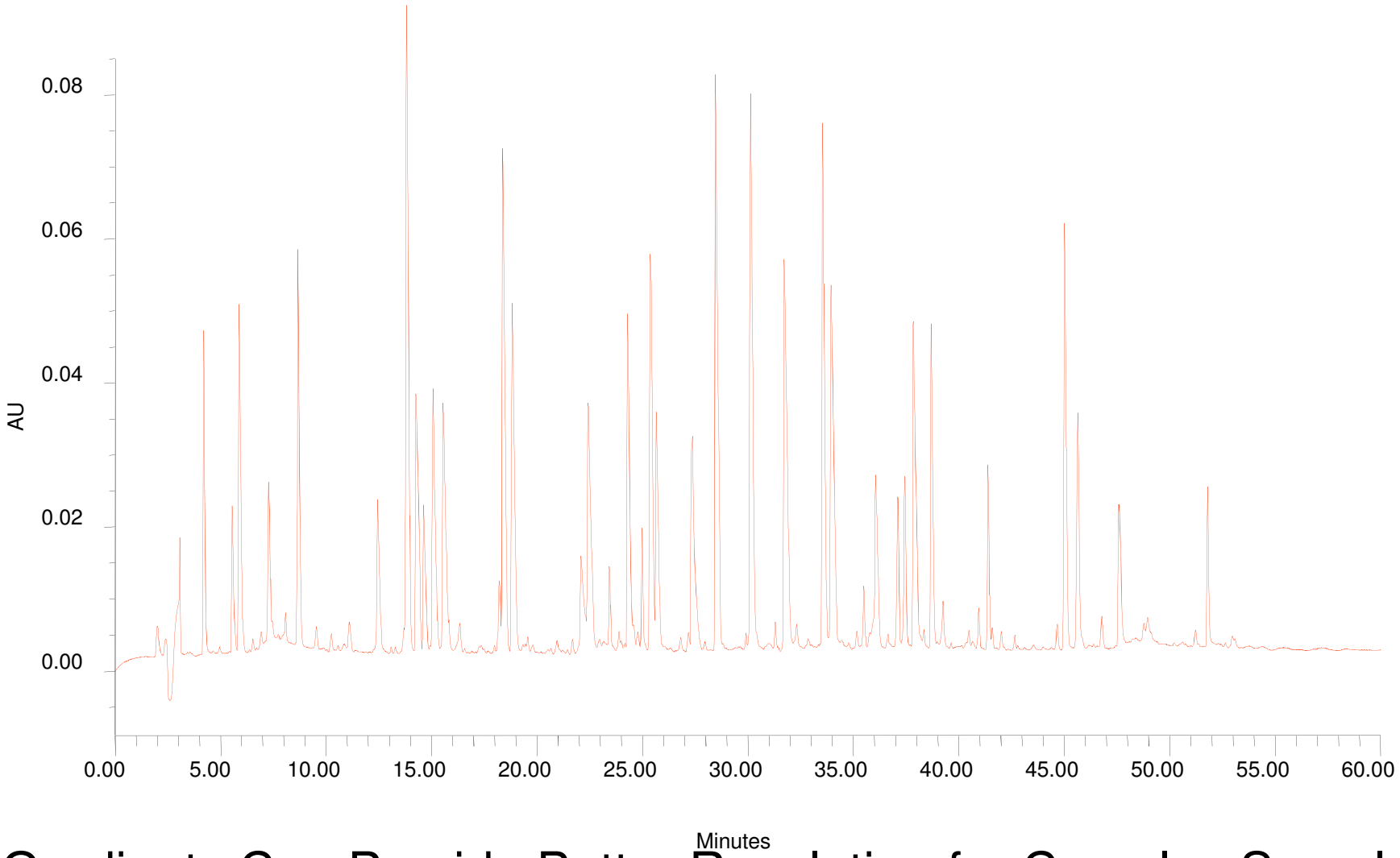

Gradients Can Provide Better Resolution for Complex Samples

Outline

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- **Particle Shape & Particle Size**
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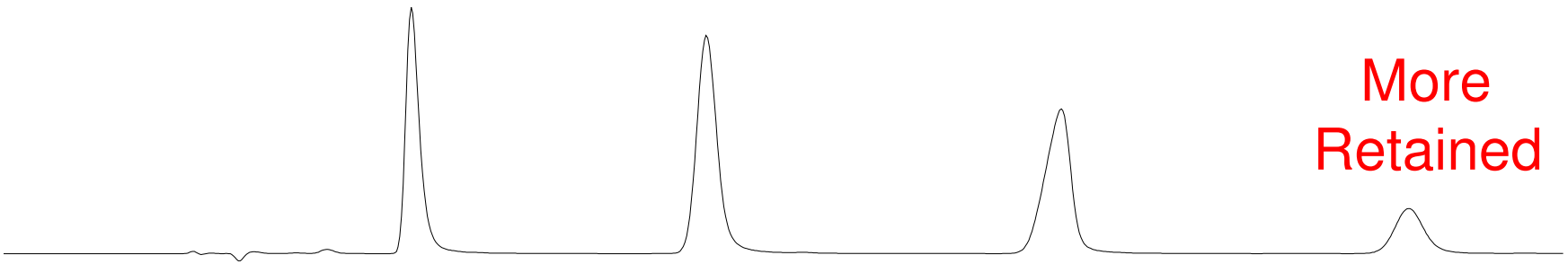
- **What is UPLC® Technology?**
- **Educational Books from Waters Corporation**

Retention Mechanisms

- **How come some peaks come out early and some come out much later ????**



Chemical Separation Power

Common modes of LC Separation

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- Polarity
  - Normal Phase
  - Reversed Phase
- Charge
  - Anion Exchange (SAX, WAX)
  - Cation Exchange (SCX, WCX)
- Size
  - Size Exclusion Chromatography (SEC)
  - Gel Permeation Chromatography (GPC)

Polarity- Characteristic of Chemicals

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Chromatographic Retention Behavior

"Like Attracts Like – Opposites are Not Attracted"

- Polars attracted to other polars (likes attract)
- Non-polars attracted to other non-polars (likes attract)
- Non-polars have no attraction to polars (opposites repel)

Chemicals are like People (Friends and Enemies)

Note: Just the reverse of magnetism, where opposites attract

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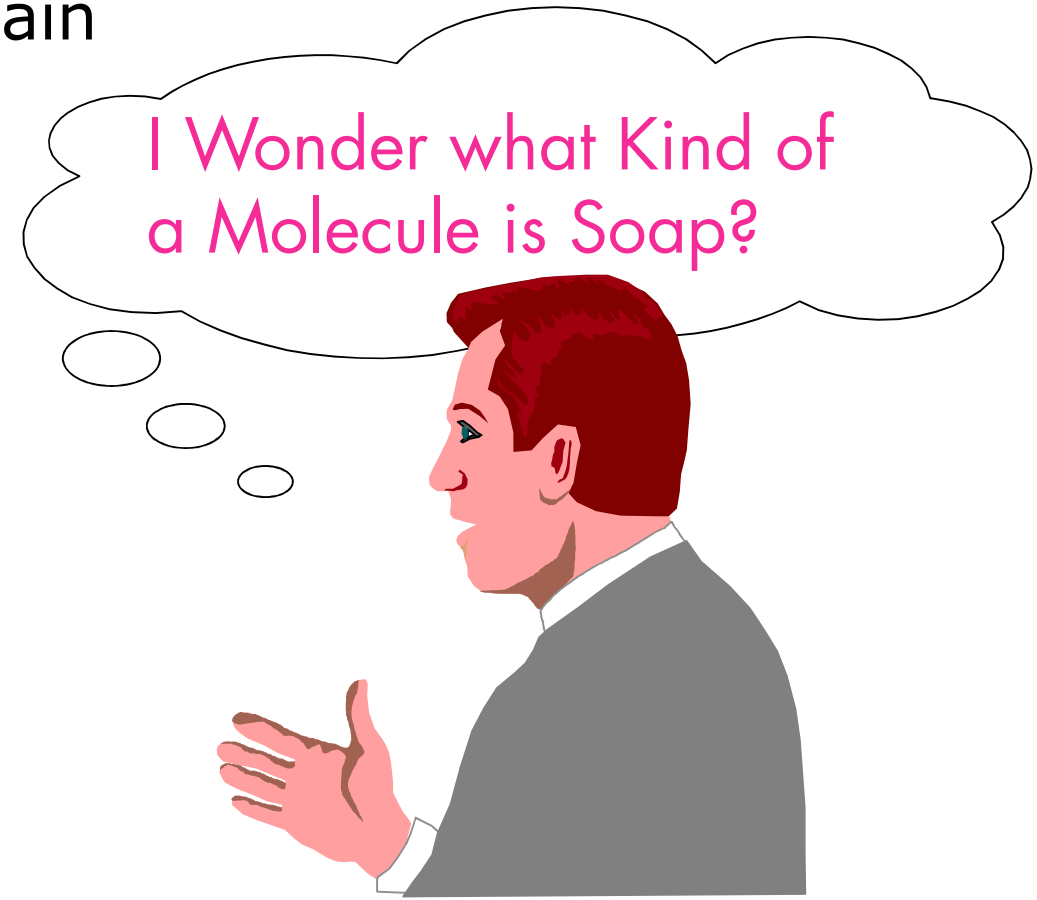
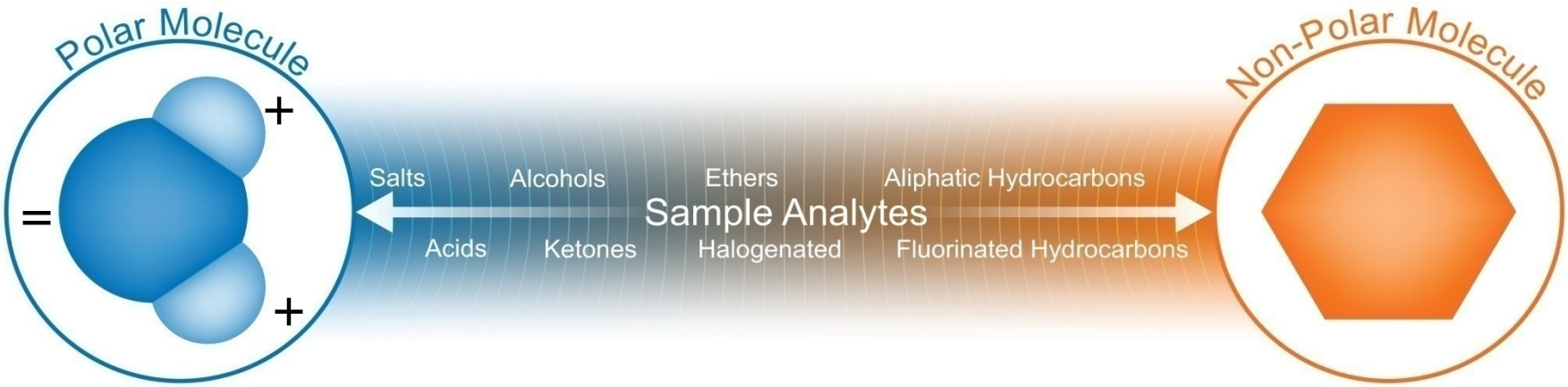
Ever try to get
Grease or Oil (Non-Polar)
off Your Hands with Plain
Water (Polar)?



Soaps and Detergents are Special Molecules that have a Polar Side AND a Non-Polar Side

Chemical Polarity*

- Characteristic of Molecules based on their Structure and Electron Charge Distribution



Scale of Polarity

* Basis for most Chromatographic Applications

Polarity Scale - Compound/Analyte

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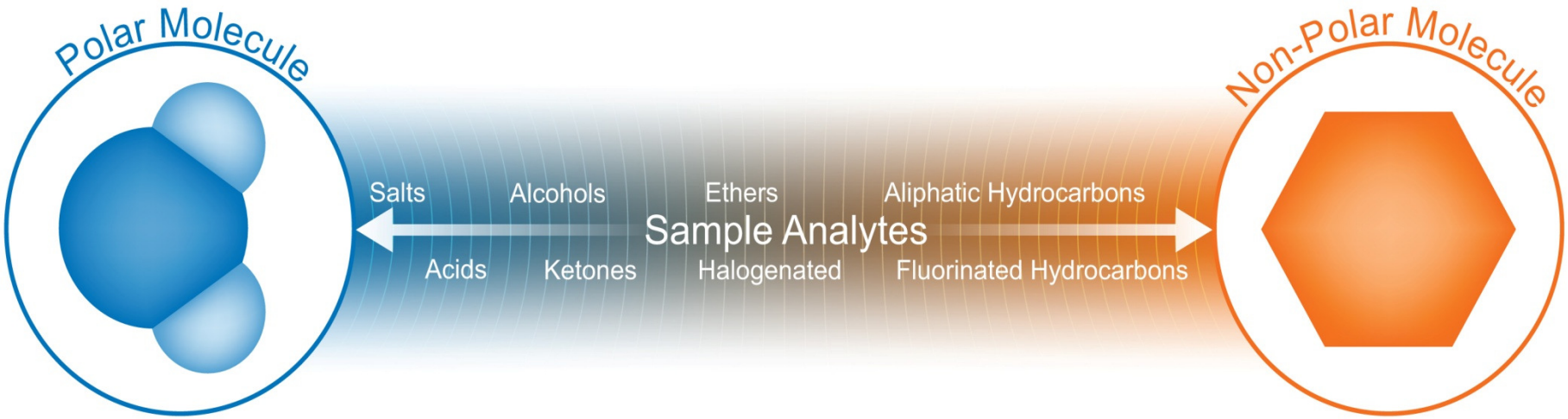

Polarity Scale- Mobile Phase

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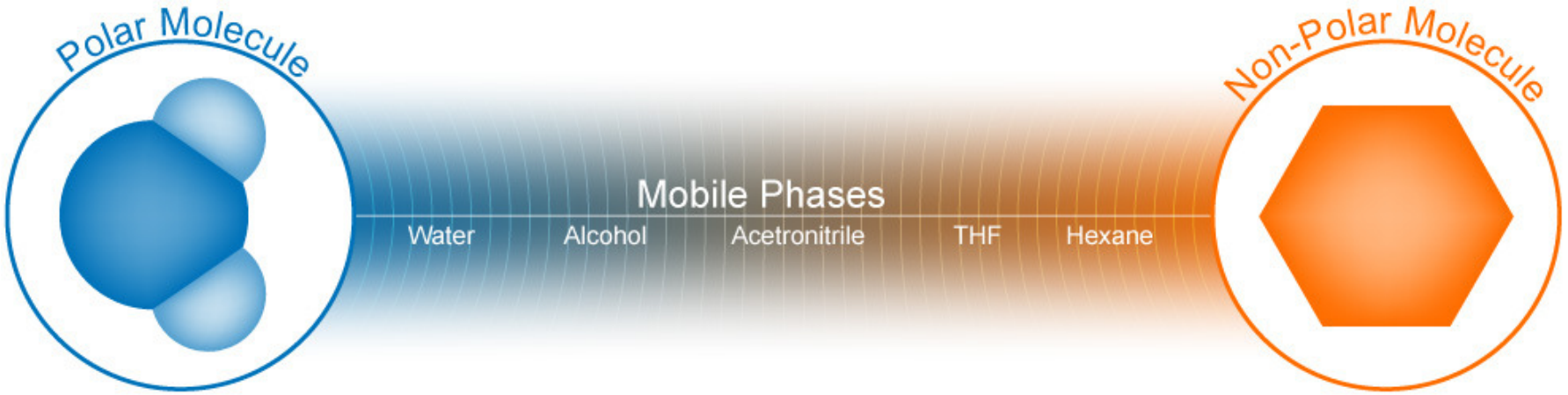

Polarity Scale- Particle/Stationary Phase

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Un-bonded Silica

C18 Bonded Silica

Competition between the Stationary Phase and the Mobile Phase for different analytes creates a separation – changes the rates of speed of the analytes based upon the different attractions – this competition occurs on the chromatographic surface of the particle (stationary phase) wetted by the mobile phase.

Polarity - Characteristic of Chemicals

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Chromatographic Retention Behavior

"Like Attracts Like – Opposites are Not Attracted"

- Polars attracted to other polars (likes attract)
- Non-polars attracted to other non-polars (likes attract)
- Non-polars have no attraction to polars (opposites repel)

We set up a COMPETITION for the analytes by having the Mobile Phase and the Stationary Phase/Packing Material with DIFFERENT Polarities – analytes attracted to the MOBILE PHASE will be moving FASTER, while the analytes attracted to the STATIONARY PHASE/PACKING MATERIAL will SLOW DOWN

Note: Just the reverse of magnetism, where opposites attract

Chromatography Mode
Normal-Phase

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- Retention mechanism:
  - Polarity
- Stationary Phase is Polar (hydrophilic) (e.g. silica)
- Mobile Phase is Non-Polar (hydrophobic) (e.g. hexane)
- Polar analytes
  - More attracted to the polar **stationary phase**
  - Less attracted to the non-polar mobile phase
    - More retention on normal-phase column
- Non-Polar analytes
  - More attracted to the non-polar **mobile phase**
  - Less attracted to polar stationary phase
    - Less retention on normal phase column
    - FINAL RESULT: Non-Polar analytes come out before polar analytes

Unbonded Silica Gel Particle: Porous Surface

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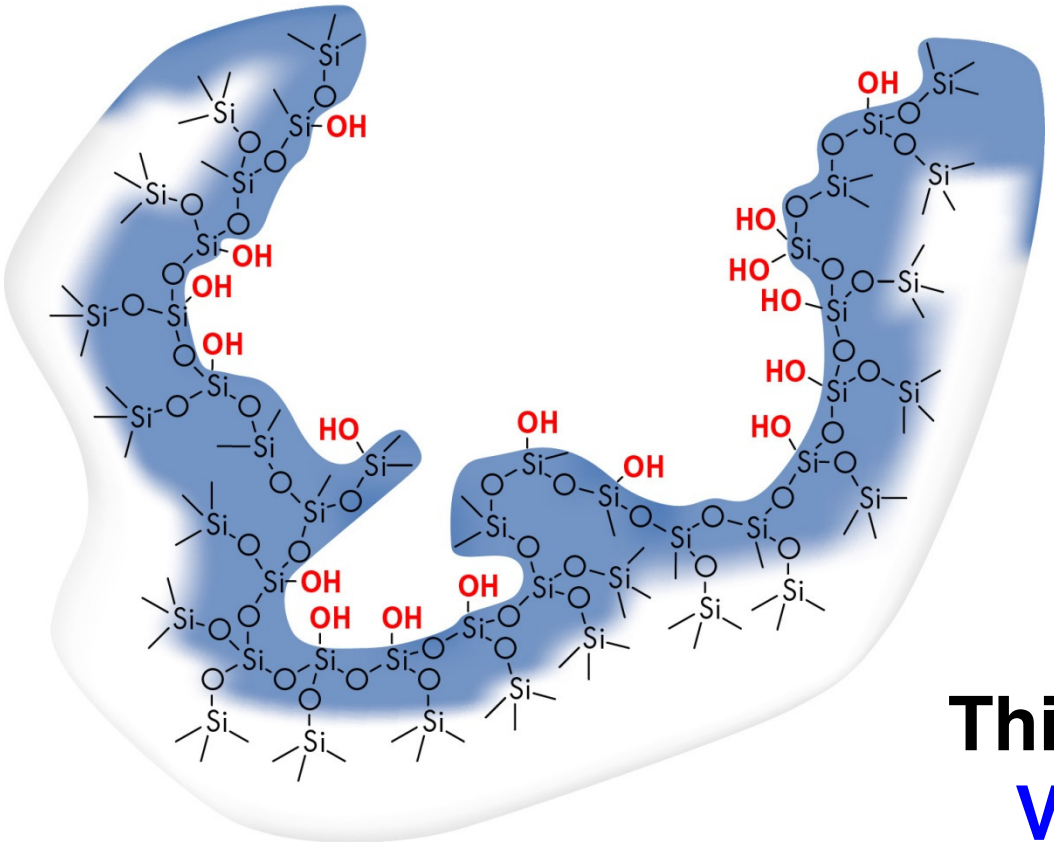

This would be

Very Polar

(Normal Phase Material)

Porous Structure of Chromatographic Particle

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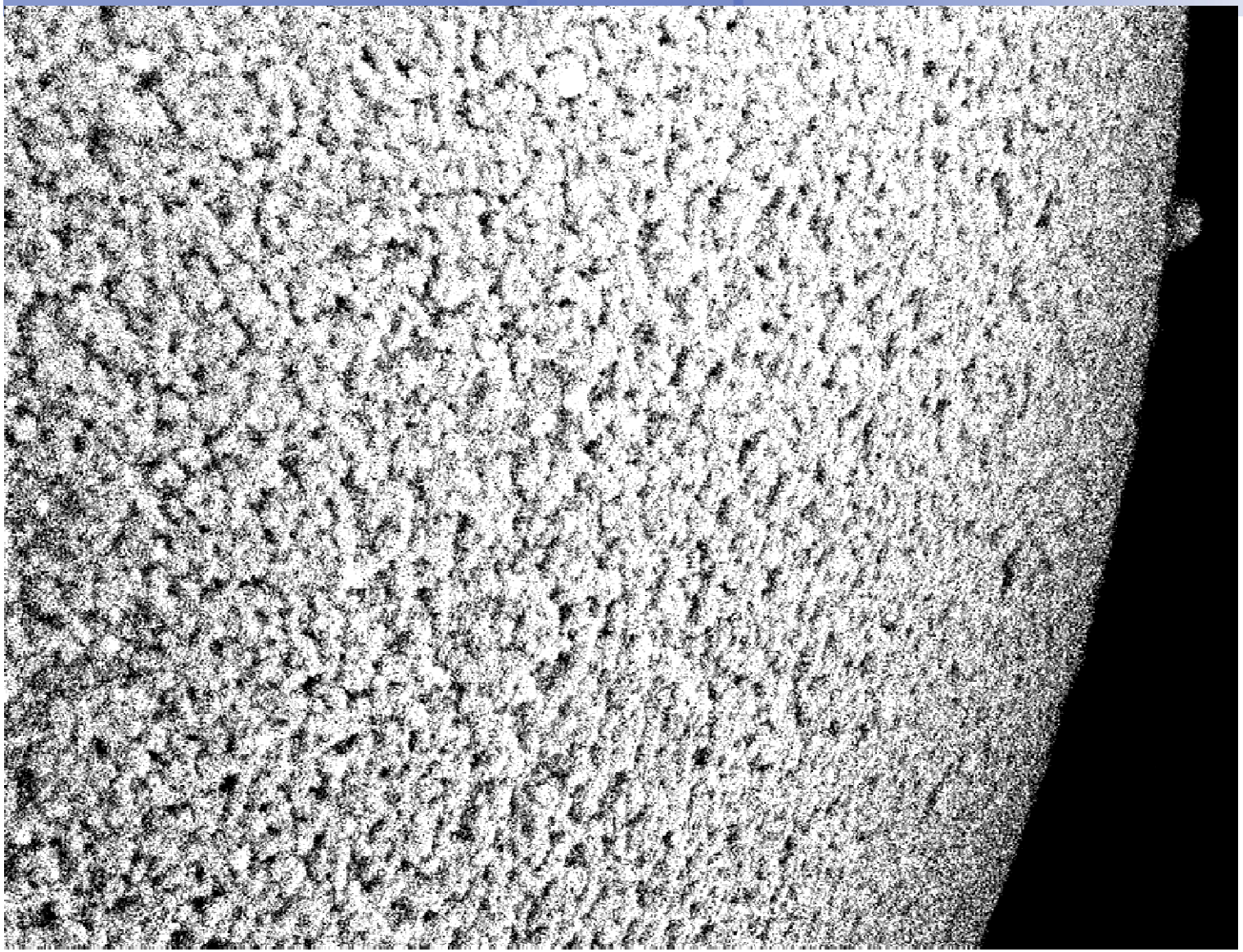

Creates a lot of Surface Area so that Separations can be made

Some Analytes go in the pores and Slow Down

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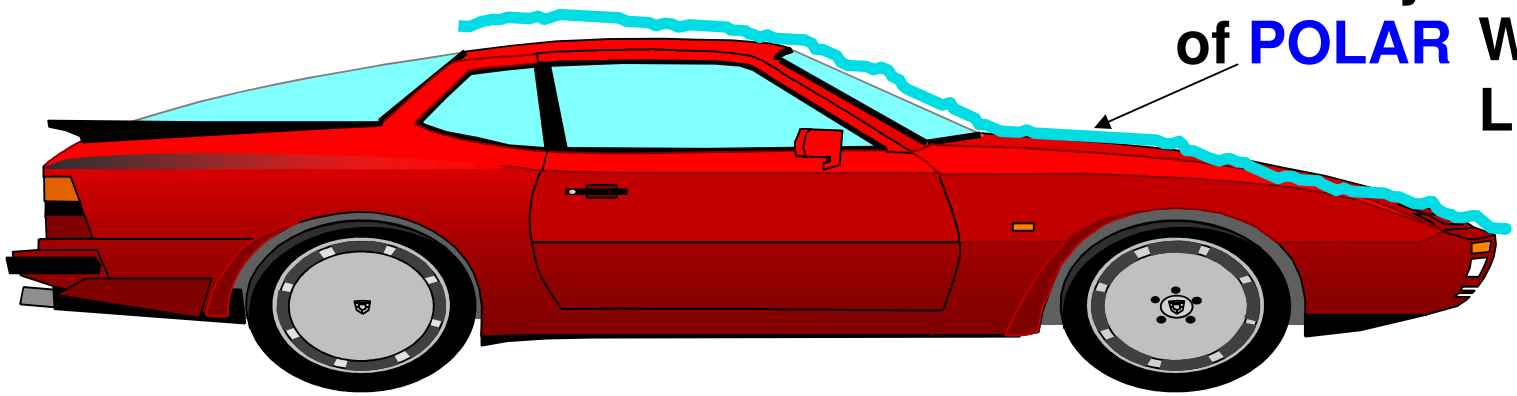
Look What Happens When It Rains On My Sports car, Which Has NOT Been Waxed In 4 Years! [A Very Polar Surface]

Rain Water Is Very Polar



Thin Shinny Film of POLAR Water - LIKES

POLAR Car Surface

Chromatography Mode
Normal Phase

Waters
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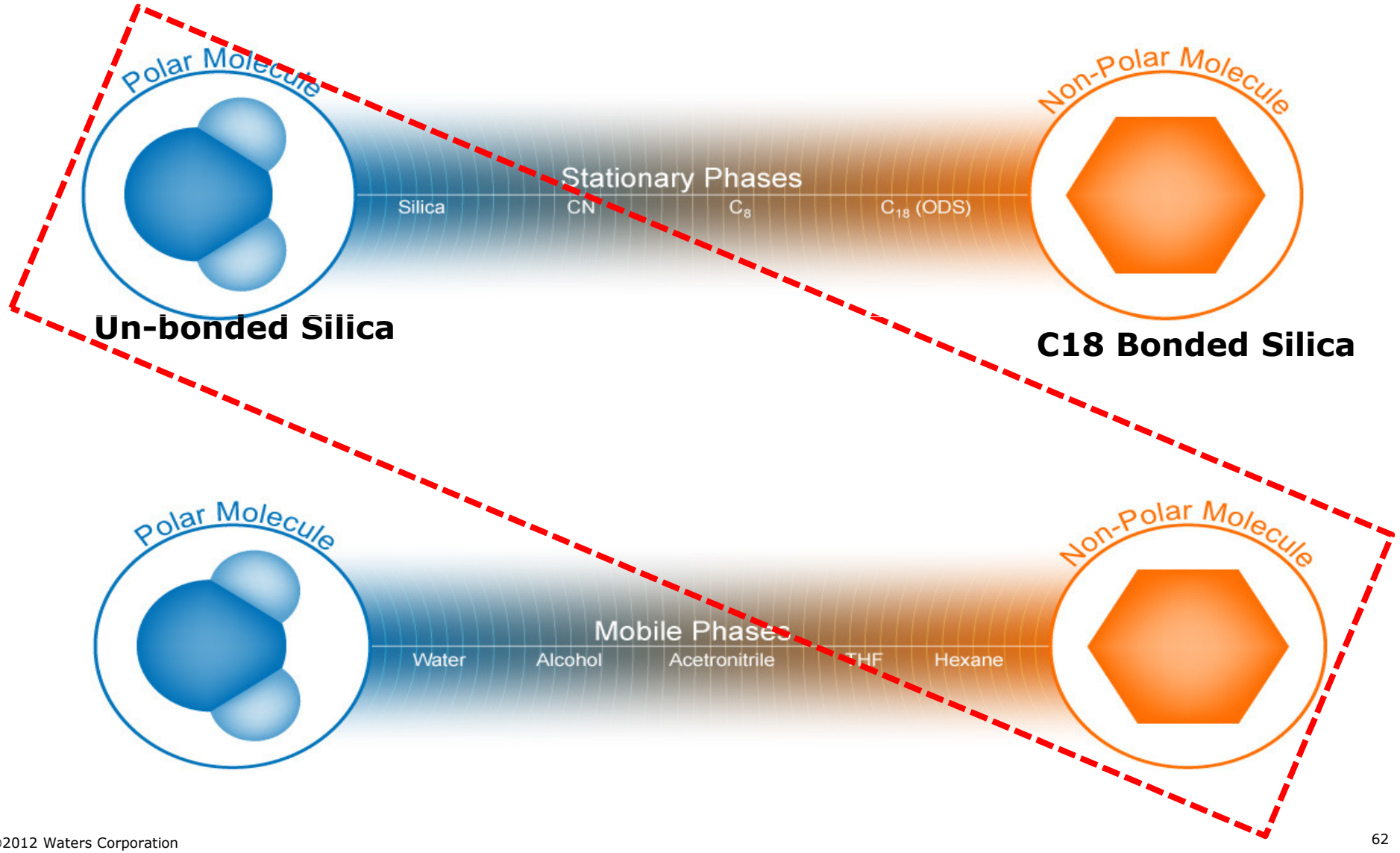

Normal-Phase Chromatography (Tswett's Experiment)

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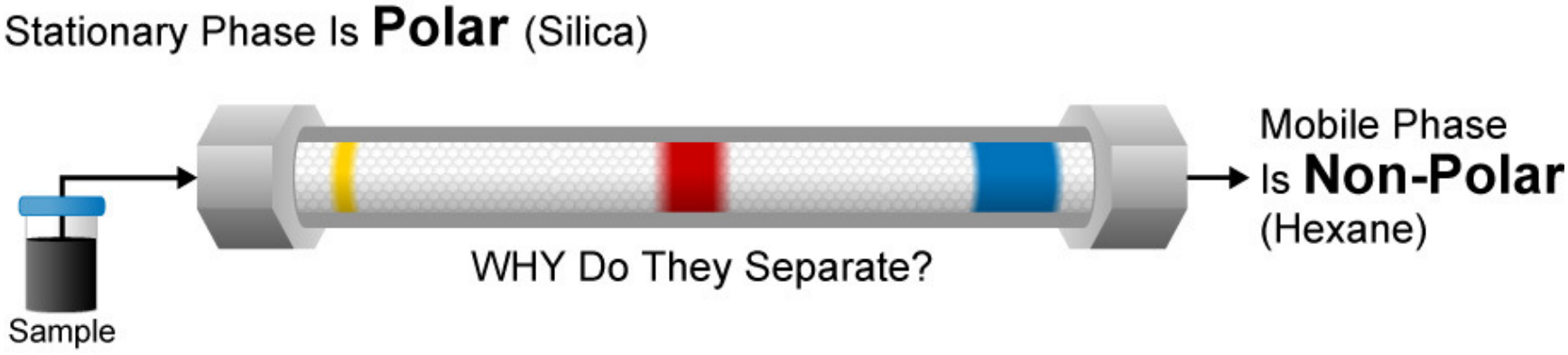

- Blue is non-polar = likes the non-polar mobile phase best, moves the fastest and comes out FIRST
- Red is moderately polar = likes the stationary phase somewhat, and slows down some
- Yellow is very polar = likes the polar stationary phase best, slows down the most and comes out LAST

In NORMAL-PHASE Chromatography, POLARS are Retained

Chromatography Mode
Reversed-Phase

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- Retention mechanism:
  - Polarity
- Stationary Phase is Non-polar (hydrophobic) (e.g. C18)
- Mobile Phase is Polar (hydrophilic) (e.g. H_2O)
- Non-Polar Analytes
  - More attracted to the non-polar **stationary phase**
  - Less attracted to the polar mobile phase
    - More retention on reversed-phase column
- Polar Analytes
  - More attracted to the polar **mobile phase**
  - Less attracted to non-polar stationary phase
    - Less retention on reversed-phase column
    - FINAL RESULT: Polar analytes come out before non-polar analytes

Making a Bonded Phase Material: Non-Polar {Reversed-Phase}

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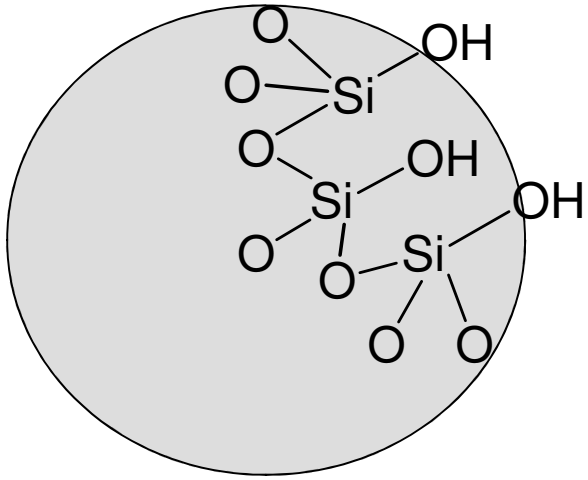
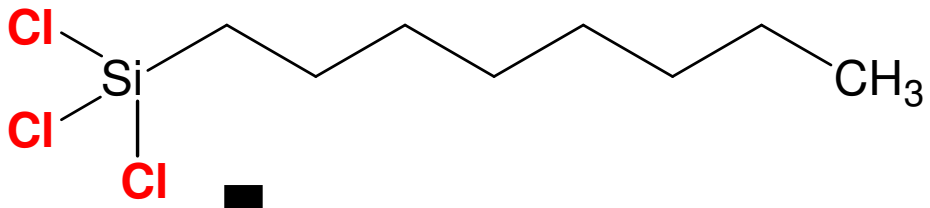
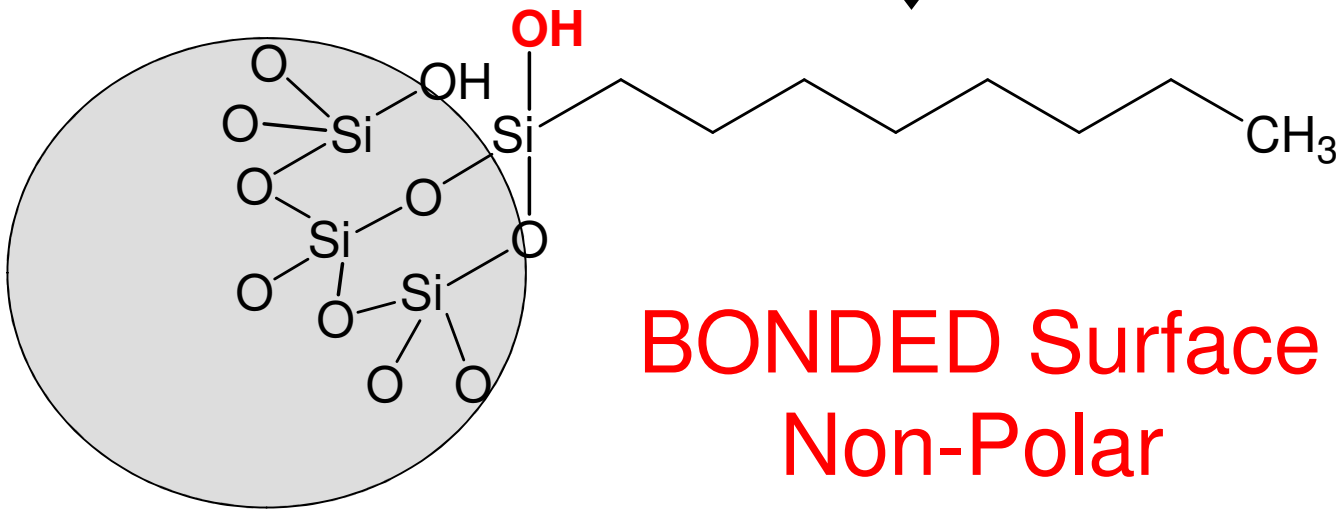
Un-bonded Silica
Polar



+

C8 Trichlorosilane "Ligand"





+ HCl

BONDED Surface
Non-Polar

C18 Bonded on Silica
Non-Polar {Reversed-Phase}

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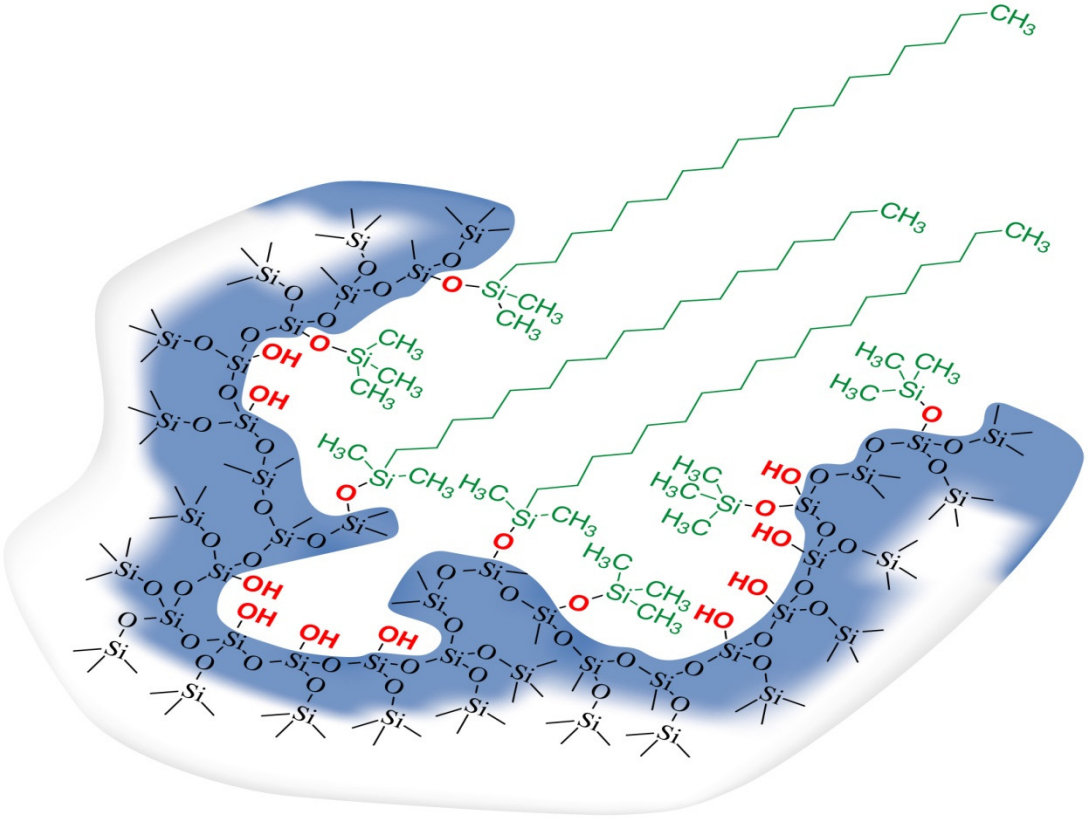
C18 (ODS) Non-Polar – Most Popular



Silica Disadvantage -- Dissolution @ High pH (that is why we invented Hybrid Particles, which are more stable ay High pH)

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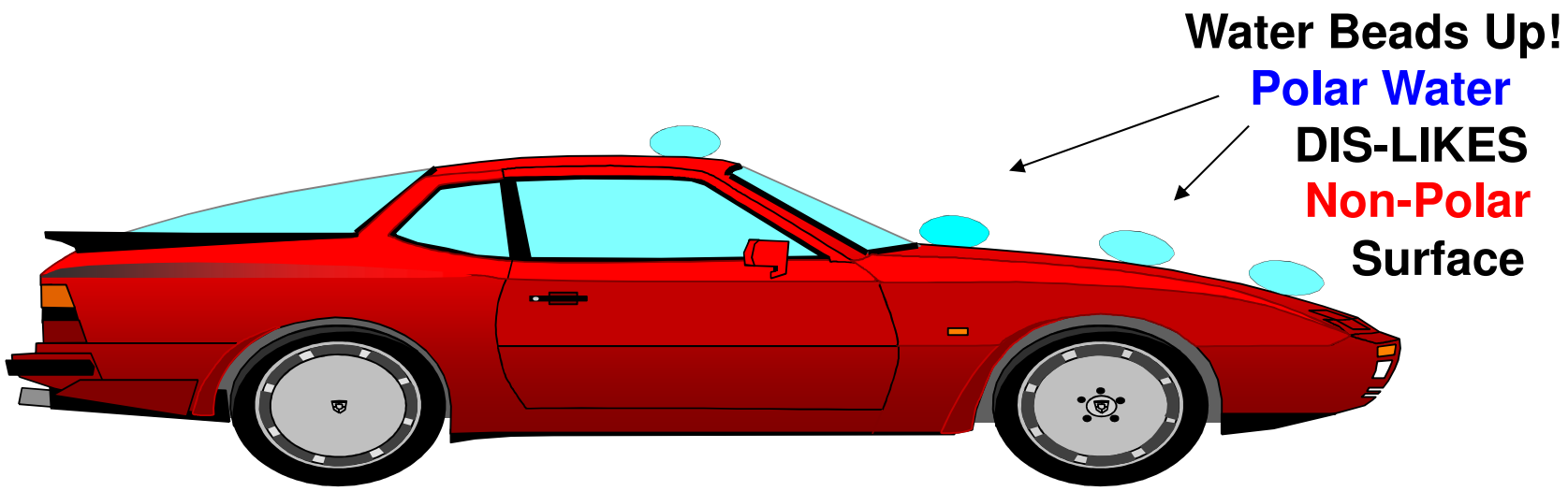
WAXING your car changes the POLAR surface to a NON-POLAR surface

Look what Happens when it Rains Right After I WAX My Car! [Non-Polar Surface]



Waxing your car is like bonding C18 to Silica – it makes it non-polar

Chromatography Mode
Reversed - Phase

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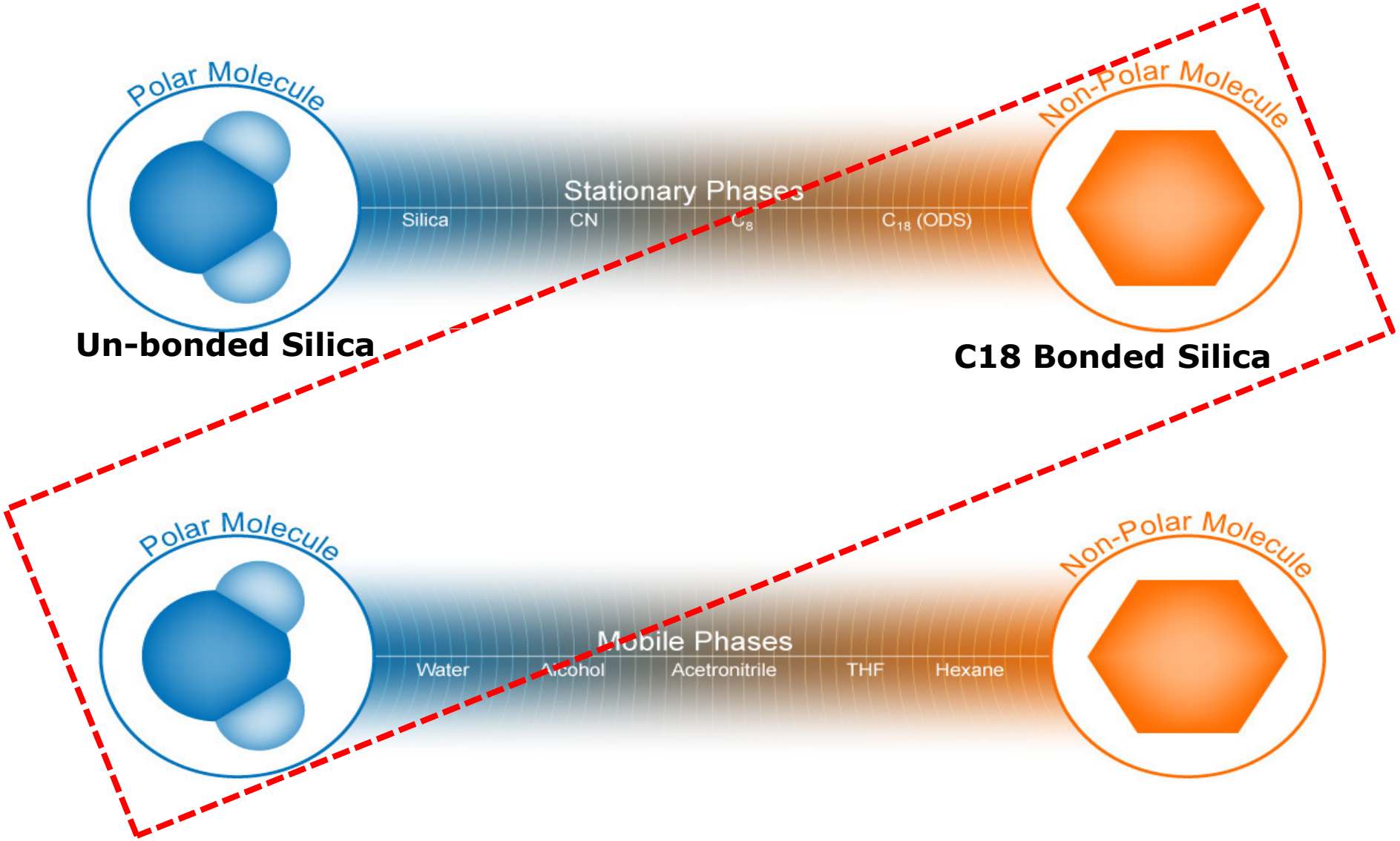

Reversed-Phase Chromatography Most Common

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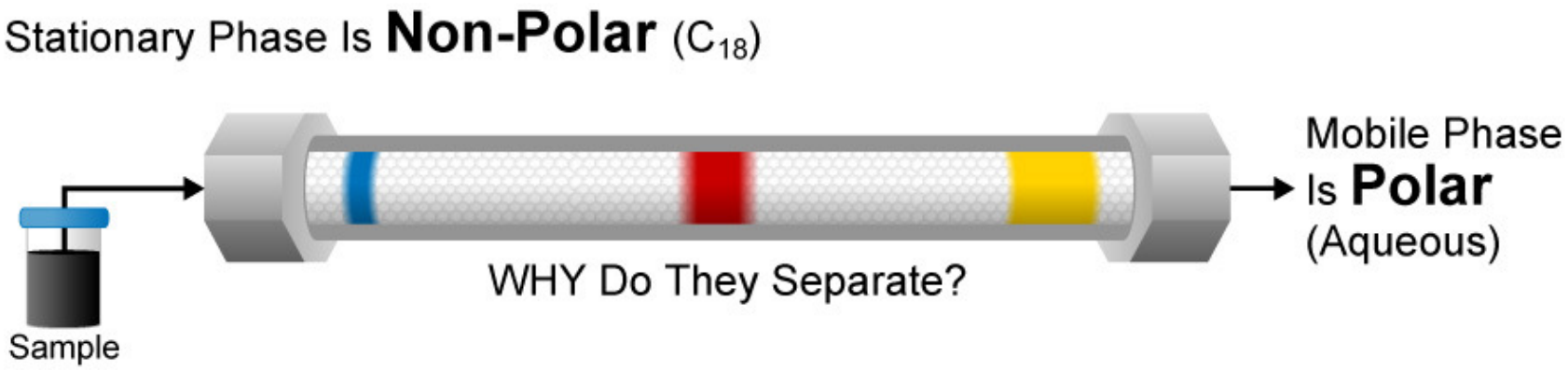
> 80% of all LC applications, and C18(ODS) is the most popular



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- Blue is non-polar = likes the non-polar stationary phase best, slows down the most and comes out LAST

In Reversed-Phase Chromatography, NON-POLARS are Retained

Chromatography Mode
Ion Exchange

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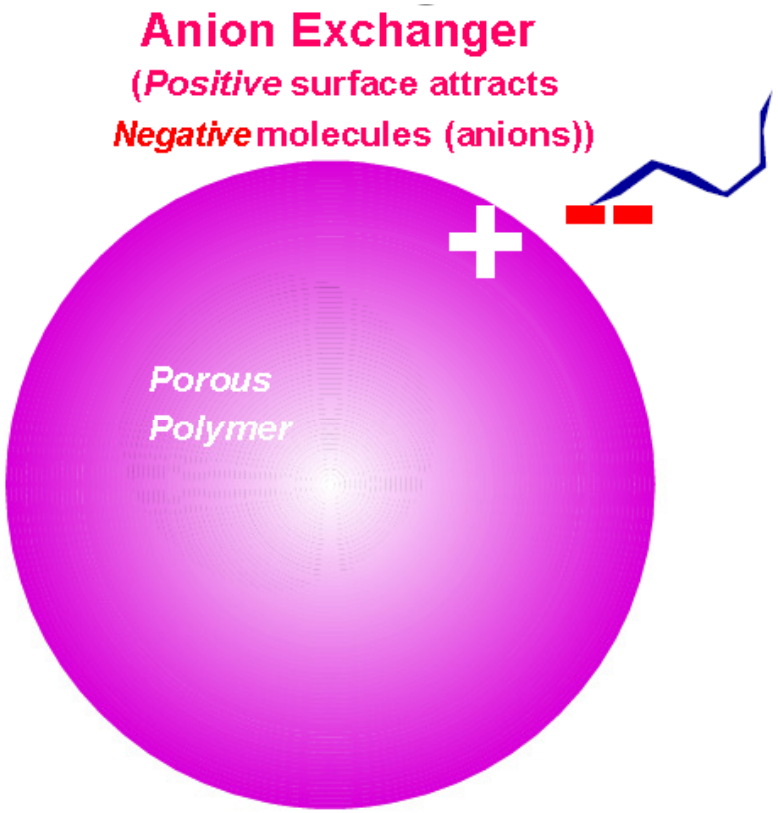
- Retention mechanism:
  - Opposite charges attract
- Anionic Molecules/Analytes
  - Have a negative charge [-]
  - Attracted to positive charge [+] of stationary phase
- Cationic Molecules/Analytes
  - Have a positive charge [+]
  - Attracted to negative charge [-] of stationary phase

Chromatography Mode
Ion Exchange



Chromatography Mode
Ion Exchange

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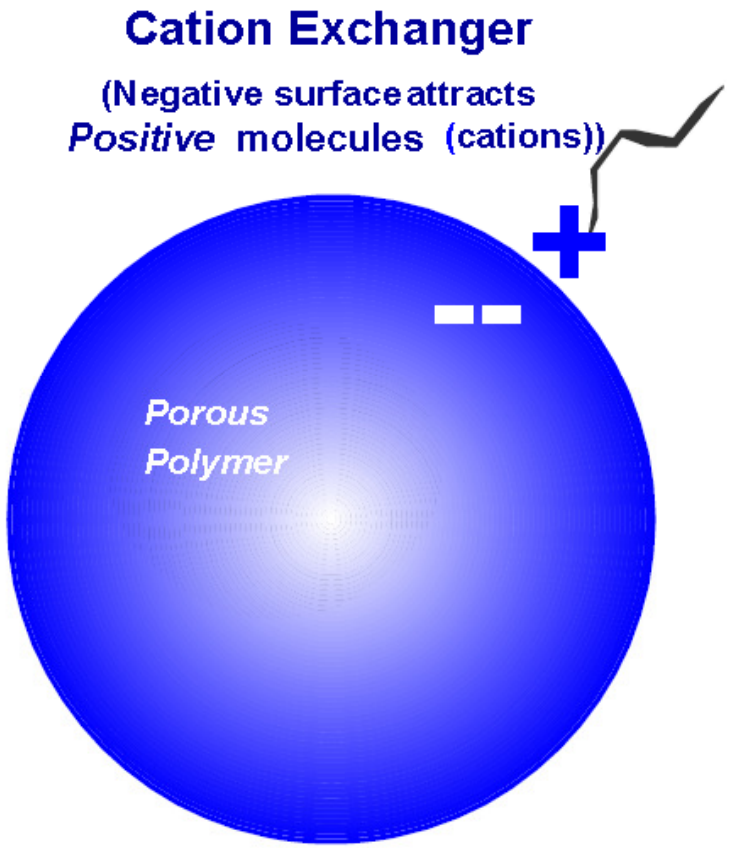

Chromatography Mode
Ion Exchange

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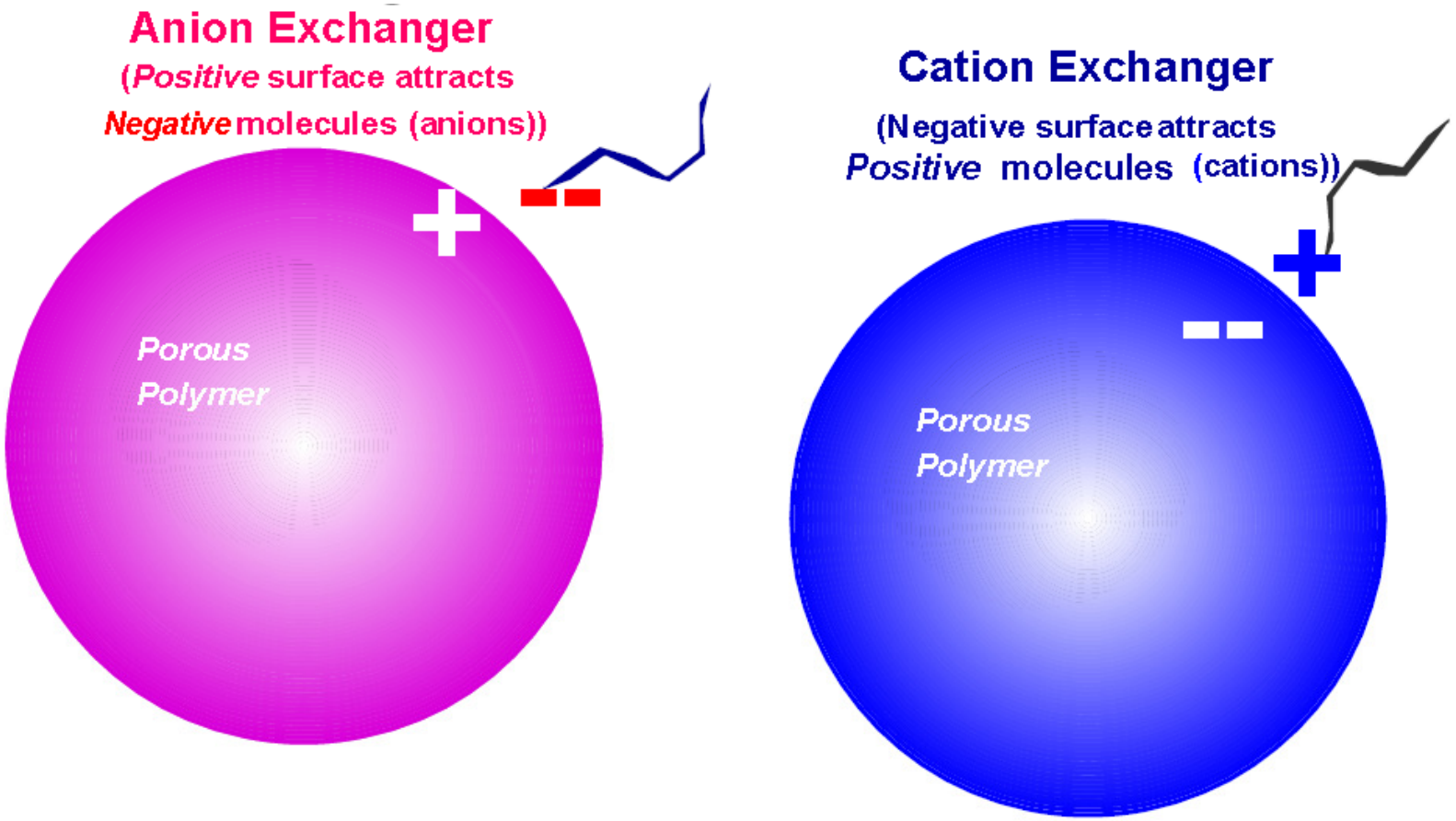

"Strong" Exchangers – ALWAYS Charged (Always On)
"Weak" Exchangers – Charged at Certain pH's ("Turn On and Off")

Chromatography Mode
SEC/GPC

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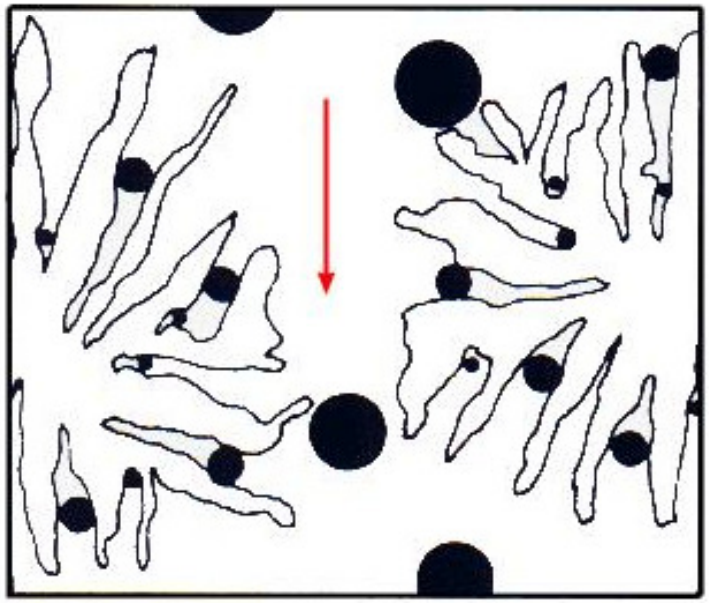
- Retention mechanism:
  - Size in solution
- Analytes are dissolved in solution
- Analytes are injected into the mobile phase (isocratic)
- Analytes are separated by their size once they are in solution

Chromatography Mode
SEC/GPC

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

- Retention mechanism:
  - Size in solution
- Analytes are dissolved in solution, injected into mobile phase
- Analytes are separated by their size once they are in solution

Cross sectional view of porous particle



Chromatography Mode
SEC/GPC

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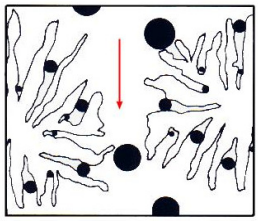
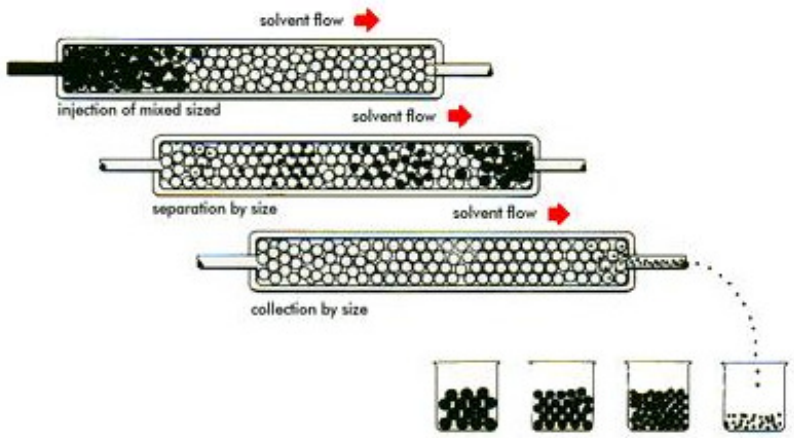
- Retention mechanism:
  - Size in solution
- Analytes are dissolved in solution, injected into mobile phase
- Analytes are separated by their size once they are in solution

Cross sectional view of porous particle



The Size Separation Mechanism



Molecules of various sizes elute from the column at different rates. The column retains low molecular weight material (small black dots) longer than the high molecular weight material (large black dots). The time it takes for a specific fraction to elute is called its "retention time".

Chromatography Mode
SEC/GPC

Waters
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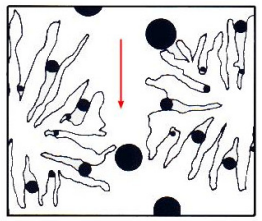
- Retention mechanism:
  - Size in solution
- Analytes are dissolved in solution, injected into mobile phase
- Analytes are separated by their size once they are in solution

- SEC (**S**ize **E**xclusion **C**hromatography)
  - generally refers to biomolecule separations.

Cross sectional view of porous particle



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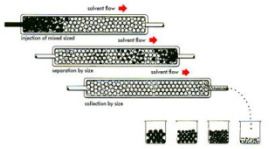

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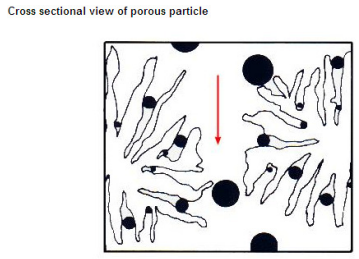

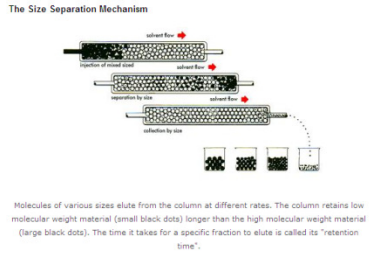

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- GPC (**G**el **P**ermeation **C**hromatography)
  - generally refers to polymer separations.

Chromatography Mode
SEC/GPC

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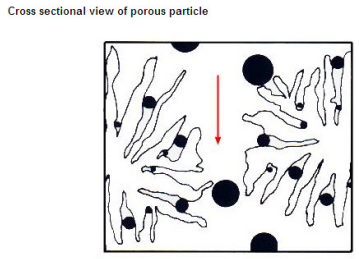

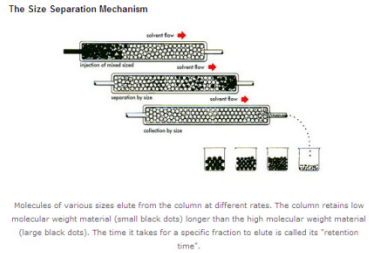

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  - generally refers to biomolecule separations.
- GPC (**G**el **P**ermeation **C**hromatography)
  - generally refers to polymer separations.
- BOCOF "**B**ig **O**nes **C**ome **O**ut **F**irst"
  - In SEC/GPC, the largest molecules come out of the column first!

Outline

Waters
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- **What is Liquid Chromatography?**
  - Chromatography Technology
  - Three Modes of Liquid Chromatography
  - What is HPLC?
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Chromatography–
Column Dimension

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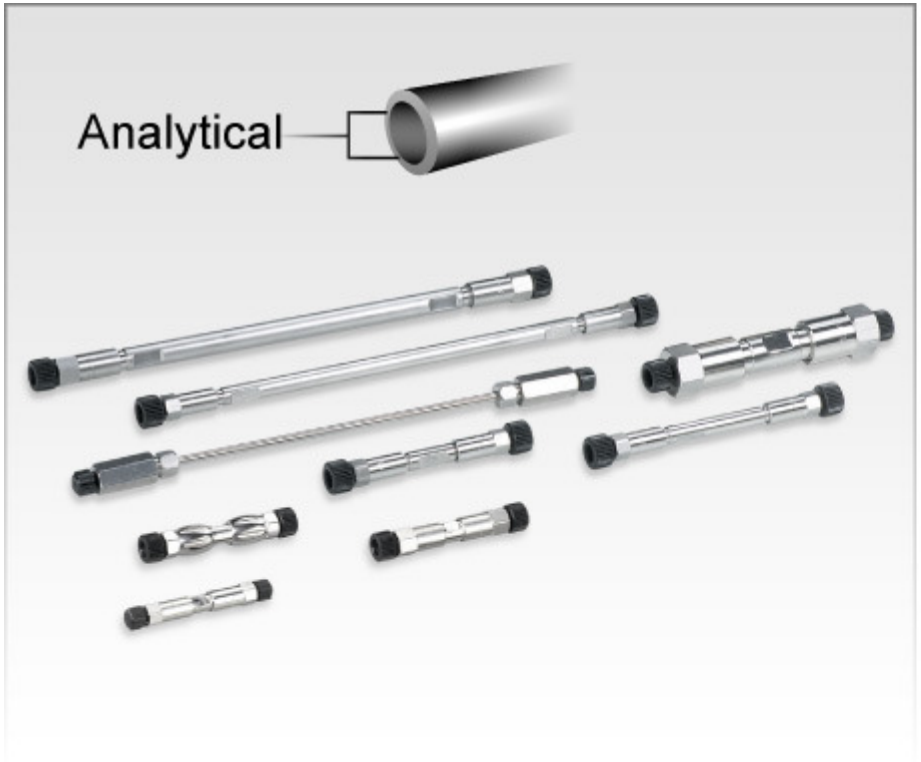

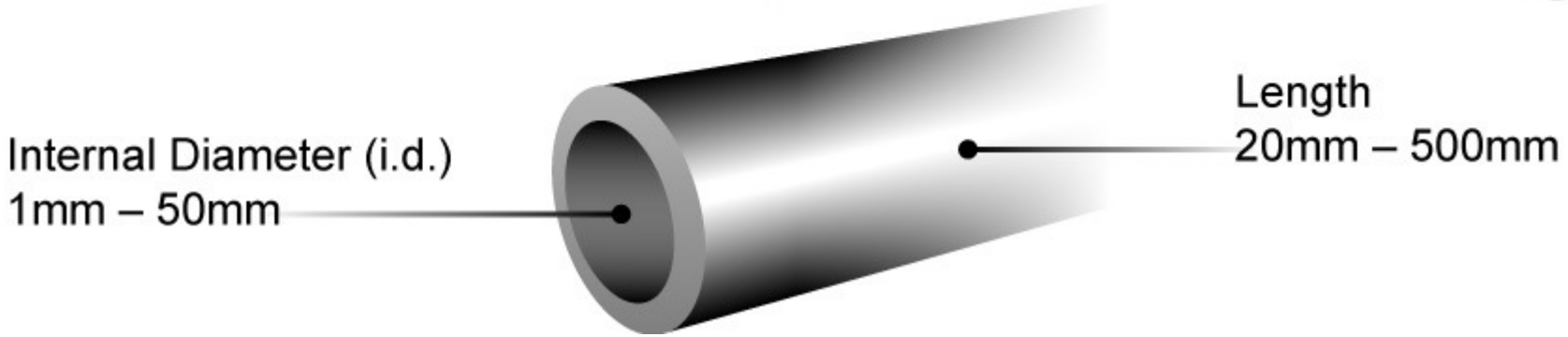

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Column Chemistry Evolution
Irregular to Spherical shaped particles

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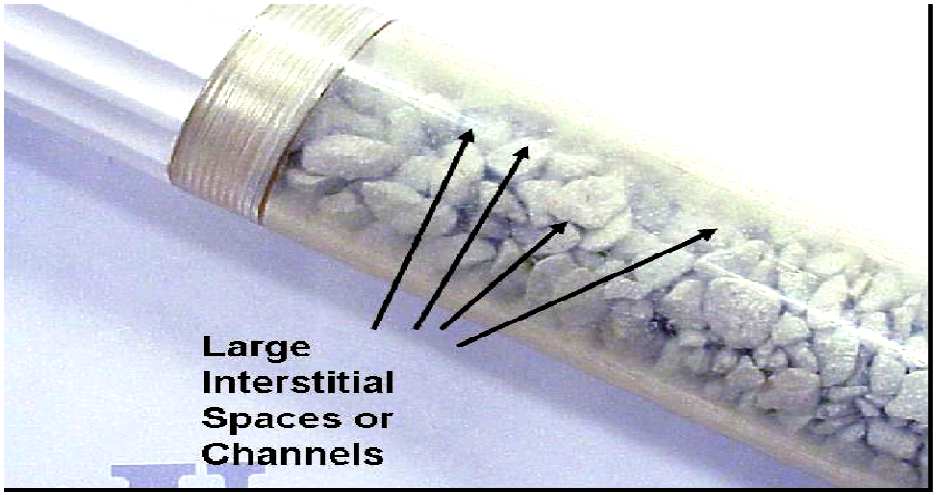
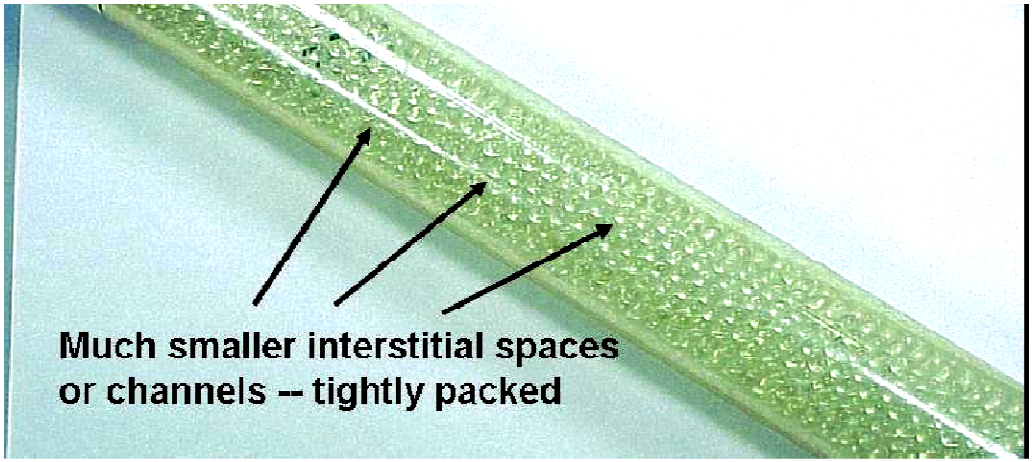
1974 helped enable technology for modern HPLC
Irregular shape, large diameter, wide particle size distribution



80's and 90's spherical shape, smaller diameter
5µm and 3µm, narrow particle size distribution



Scanning Electron Microscope
Irregular Shape

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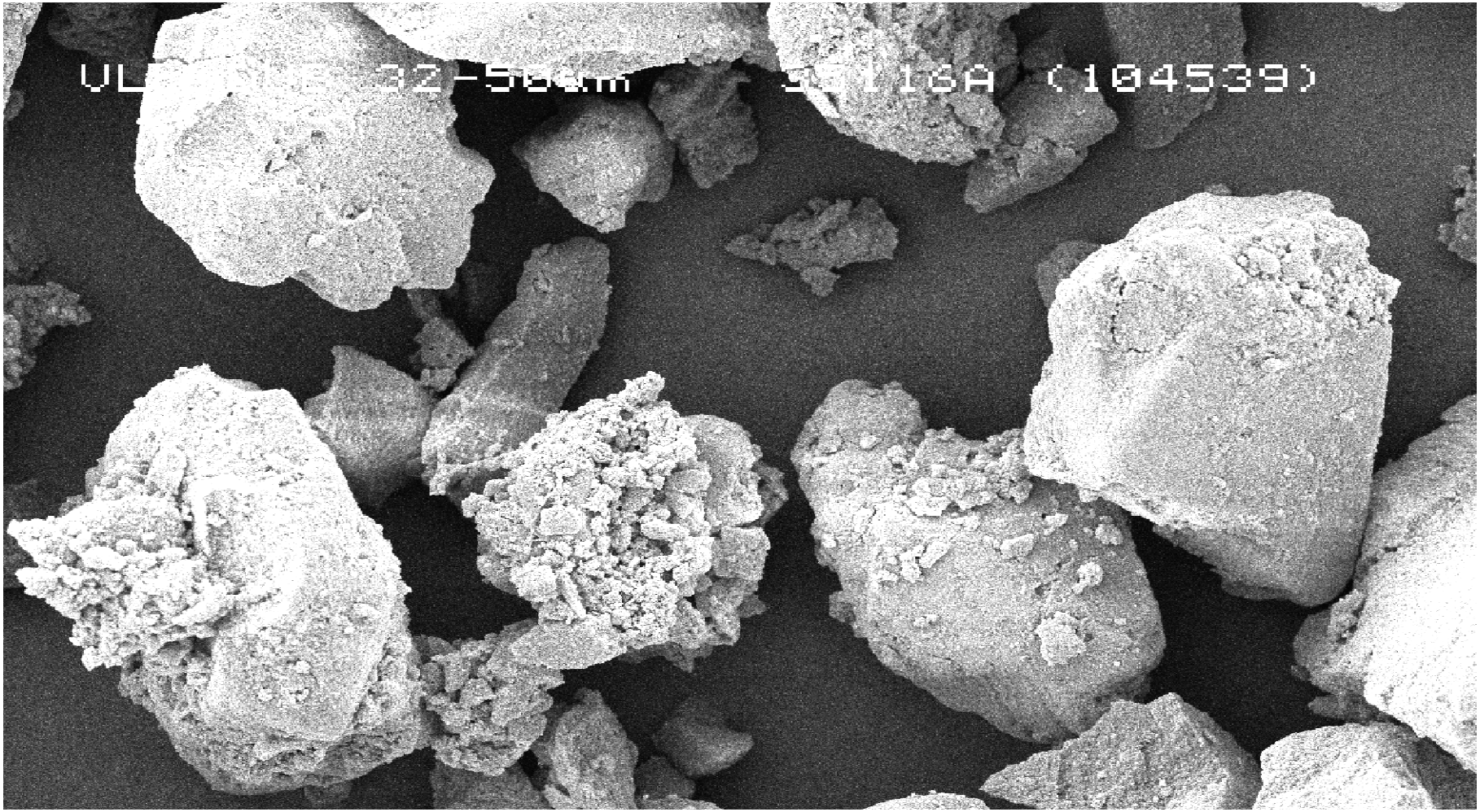

SEM
Spherical Shape

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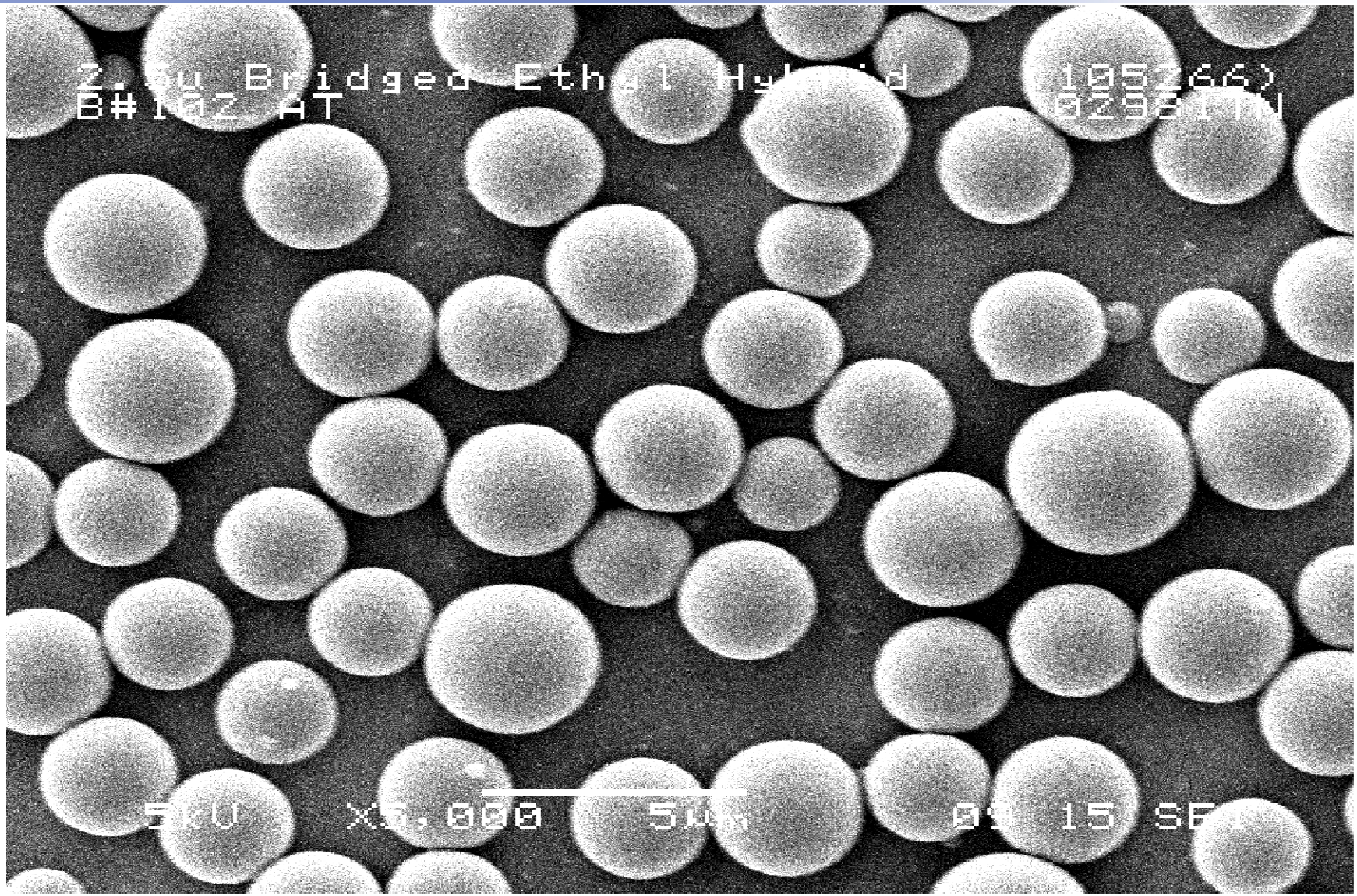

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HPLC

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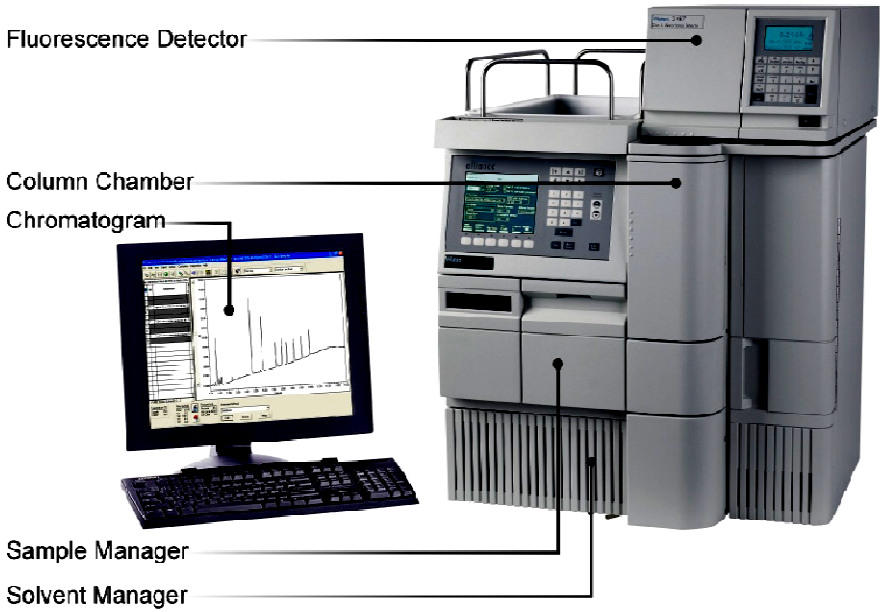
Alliance® HPLC System



Waters HPLC Columns

XBridge™ COLUMNS

XSELECT™ Columns

2.5um **XP** Columns

Atlantis® Columns

SunFire™ COLUMNS

Symmetry®

XTerra® COLUMNS

OBD™ OPTIMUM BED DENSITY PREPARATIVE COLUMNS

BioSuite™

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What is UPLC® Technology?

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UltraPerformance Liquid Chromatography (UPLC Technology):

In 2004, further advances in instrumentation and column technology were made to achieve very **significant increases** in resolution, speed, and sensitivity in liquid chromatography.

What is UPLC® Technology?

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UltraPerformance Liquid Chromatography (UPLC Technology):

In 2004, further advances in instrumentation and column technology were made to achieve very **significant increases** in resolution, speed, and sensitivity in liquid chromatography.

Columns with smaller particles [1.7 micron] and instrumentation with specialized capabilities designed to deliver mobile phase at 15,000 psi [1,000 bar] were needed to achieve a new level of performance.

What is UPLC® Technology?

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A new system had to be holistically created to perform ultra-performance liquid chromatography, now known as UPLC® technology.

UPLC® (Ultra Performance Liquid Chromatography)

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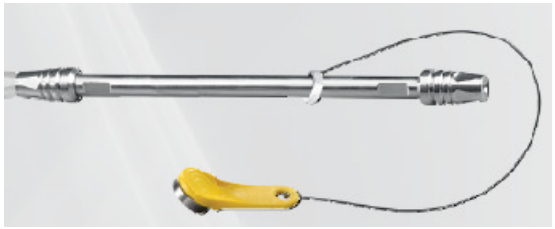
ACQUITY UPLC® System

ACQUITY UPLC® Columns



BEH Technology™

CSH™ Technology

HSS
HIGH STRENGTH SILICA

Waters Particle Technology

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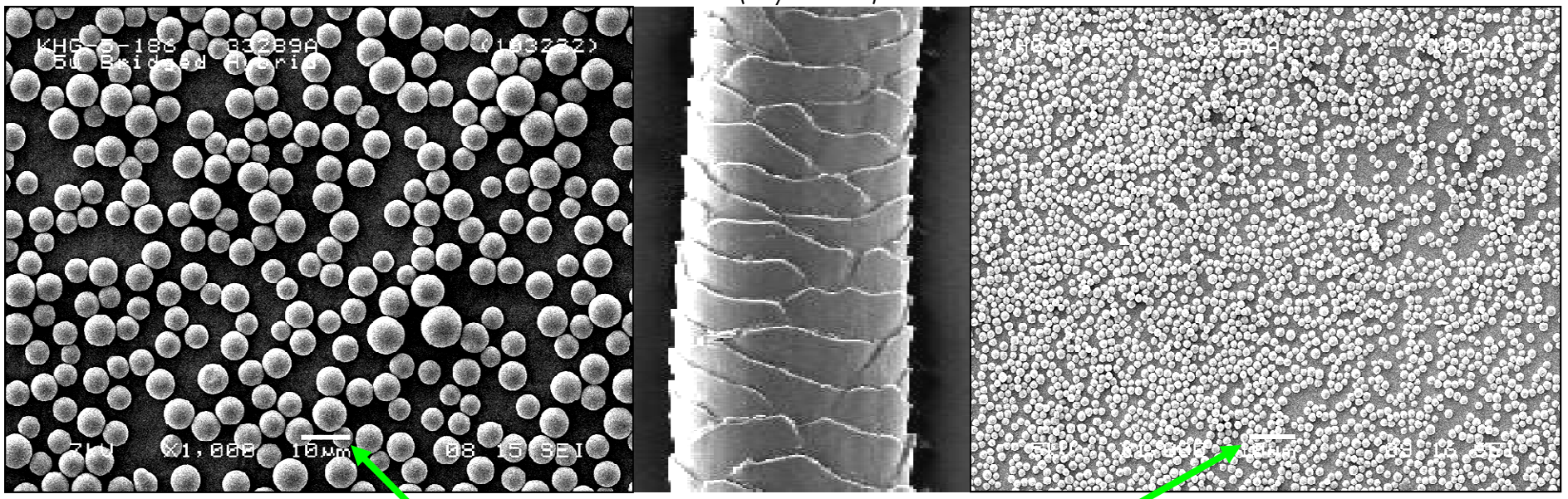
60 µm Human Hair
(very fine hair)



5 µm
Analytical Particles
(can fit 12 across hair)

1.7 µm
ACQUITY UPLC® Particles
(can fit 33 across hair)

Images are on the same scale (Bar = 10 µm)

Particle Size and Mechanical Separating Power*

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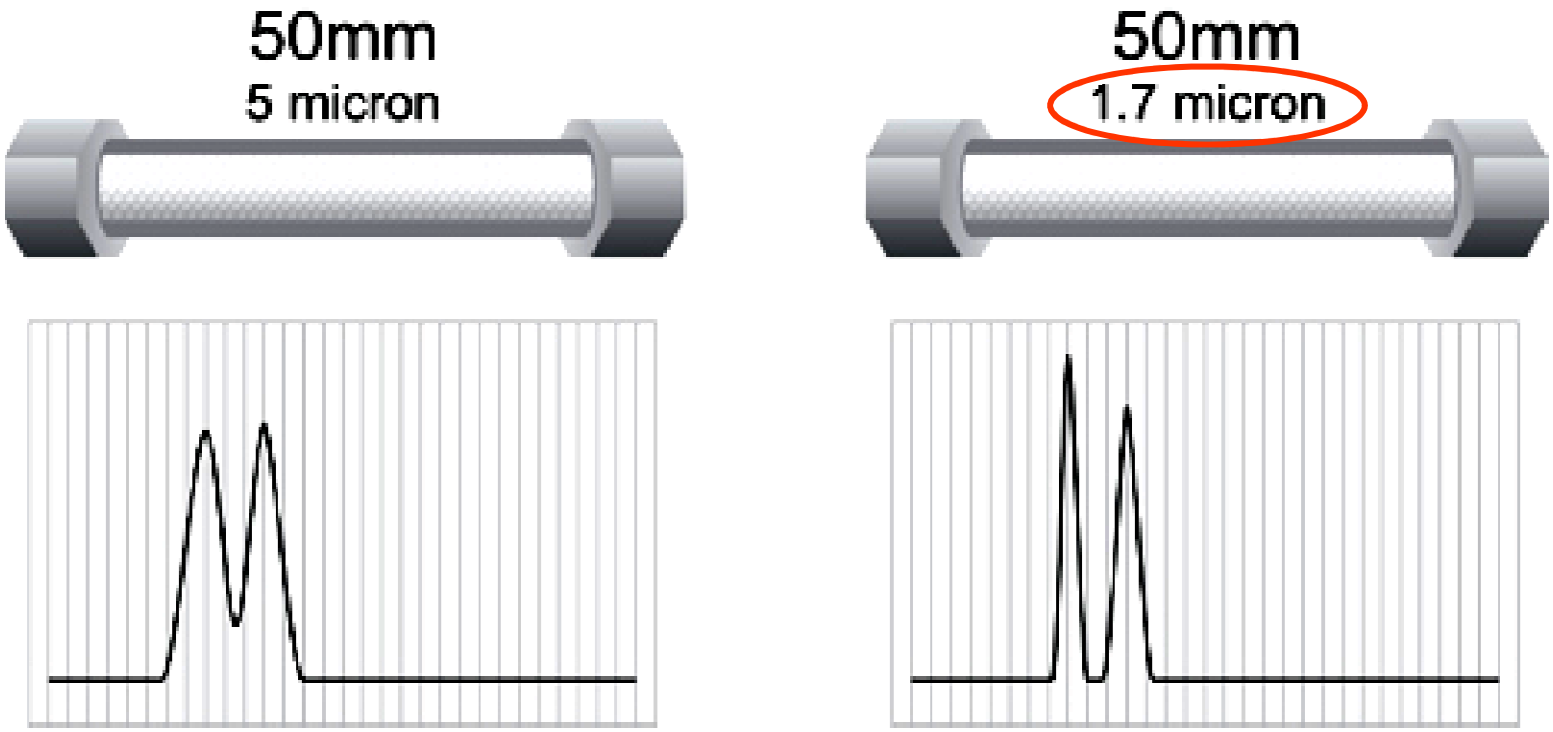
Columns contain the same packing material chemistry, are the same length with the same mobile phase. *One column has particles which are a third the size.*



Smaller particle sizes provide for a better separation with the same run time. However, back pressure will increase.

* This is also called "Efficiency"

Column Length and Mechanical Separating Power*

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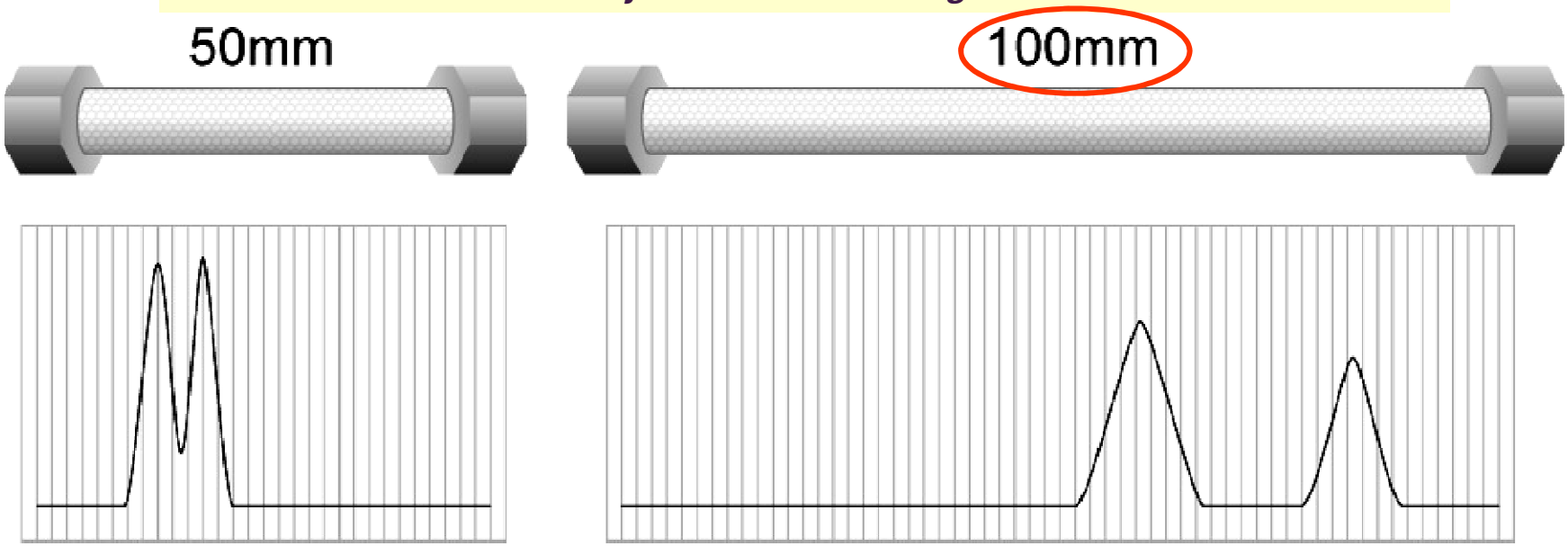
Columns contain the same packing material, same particle size and same mobile phase, *only one is twice as long*



Additional column length does provide a better separation.

However, several "costs" are incurred: ***more time (2X) for the analysis, use more solvent, increased back pressure and the longer column costs more to buy.***

A better approach, would be to try a different particle chemistry/mobile phase combination or a smaller particle size that can create the separation in less time.

* This is also called "Efficiency"

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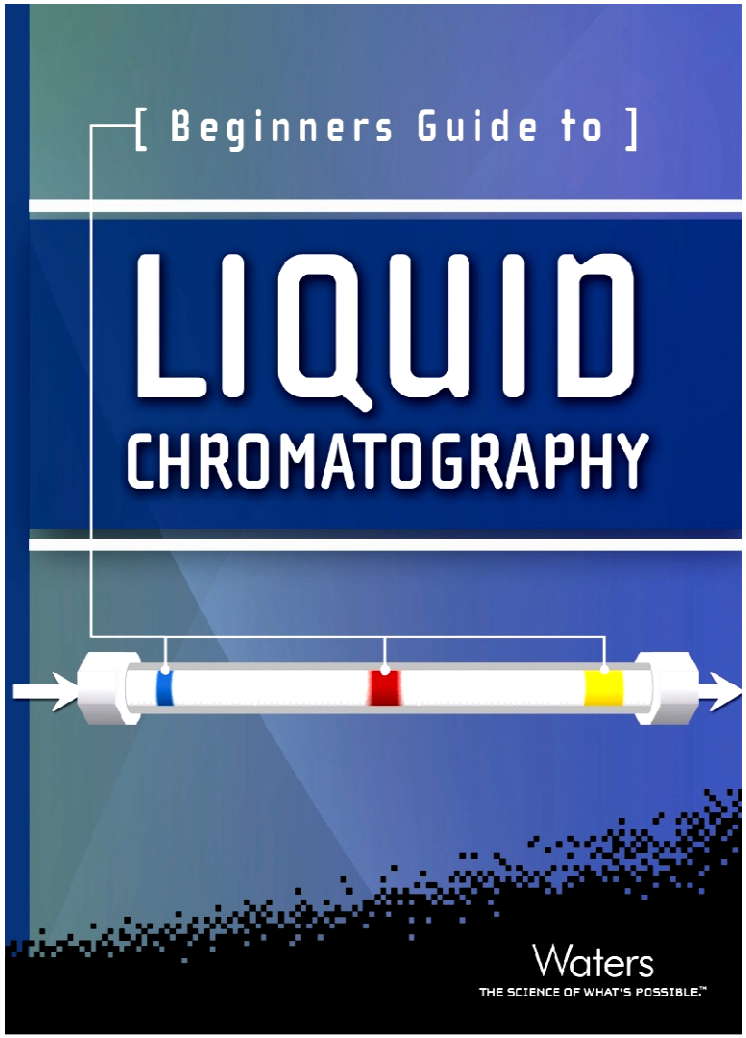
Educational Booklets



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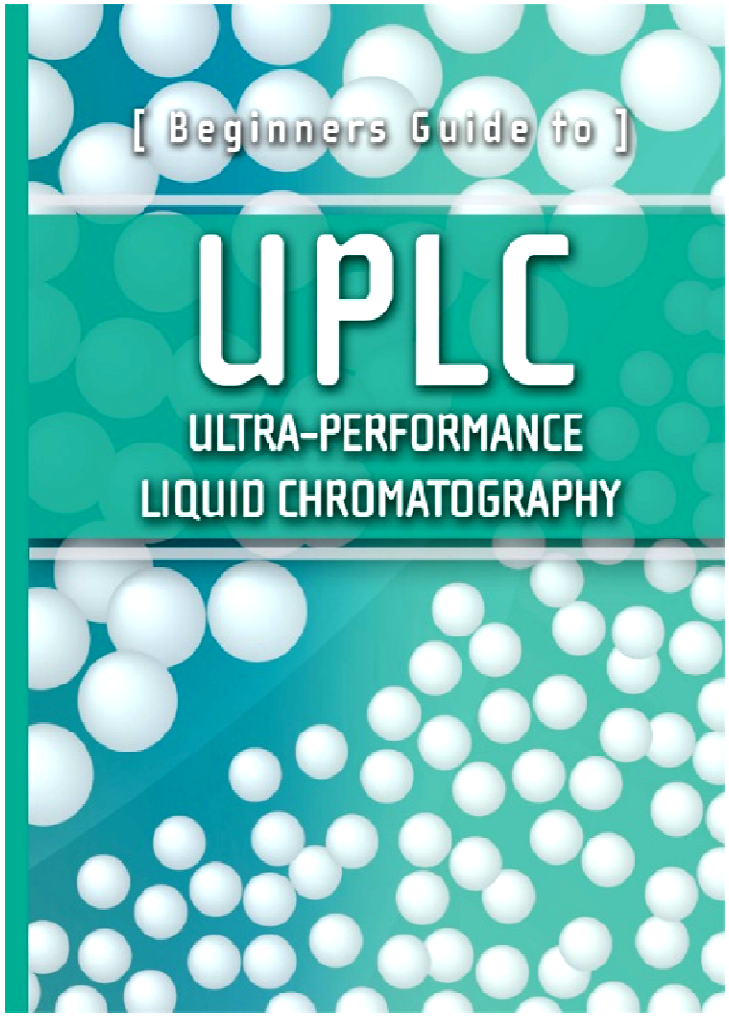

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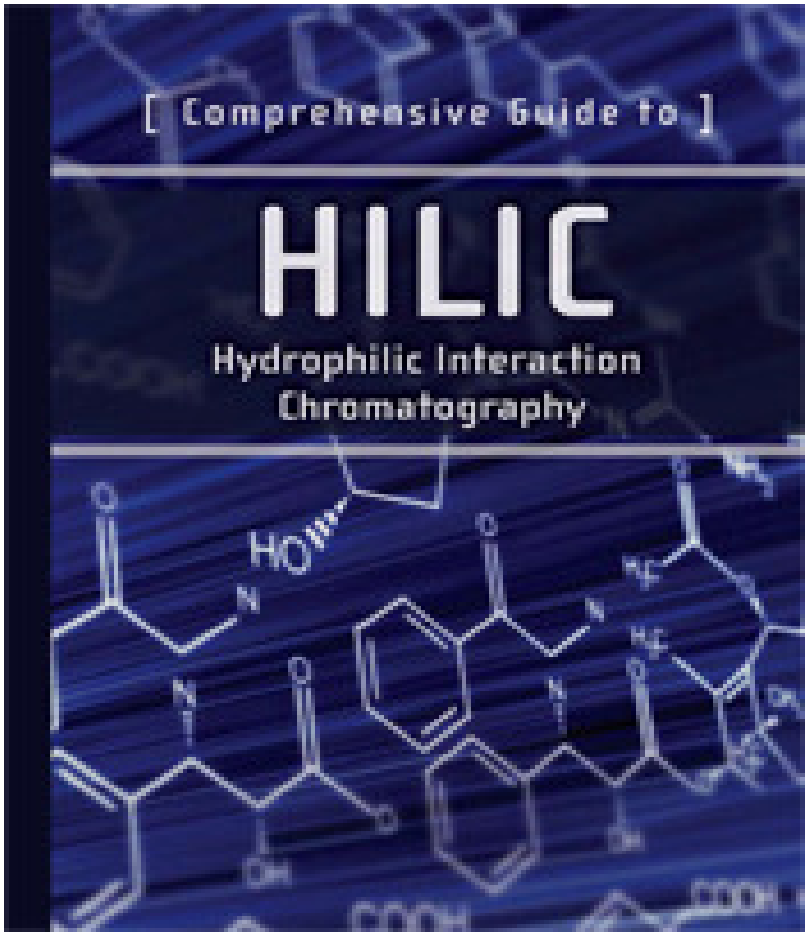

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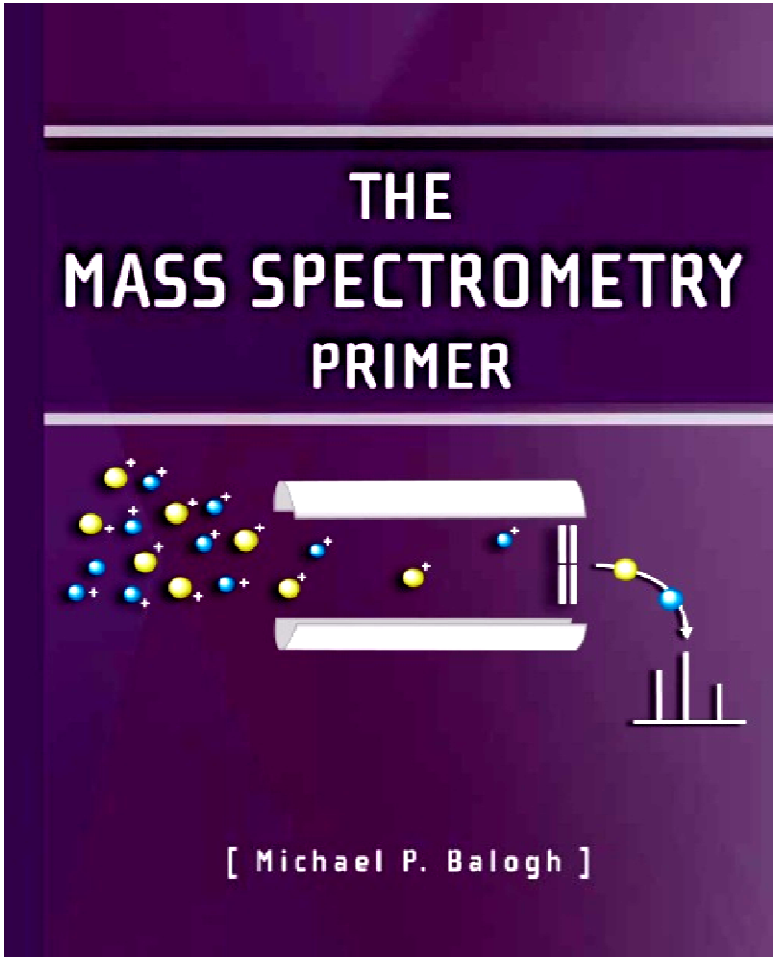

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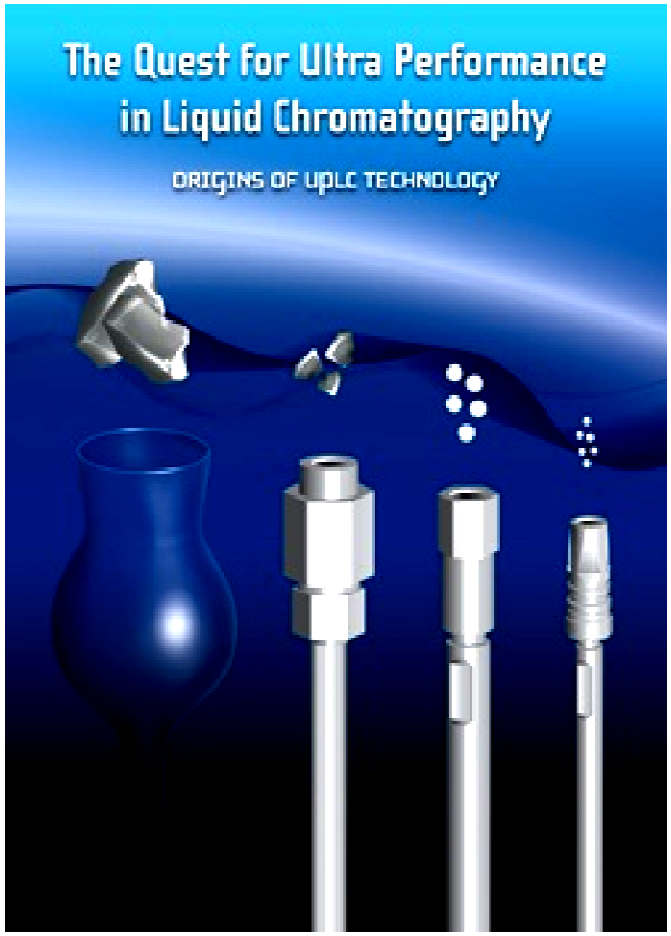

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Technology Capability Literature Primers

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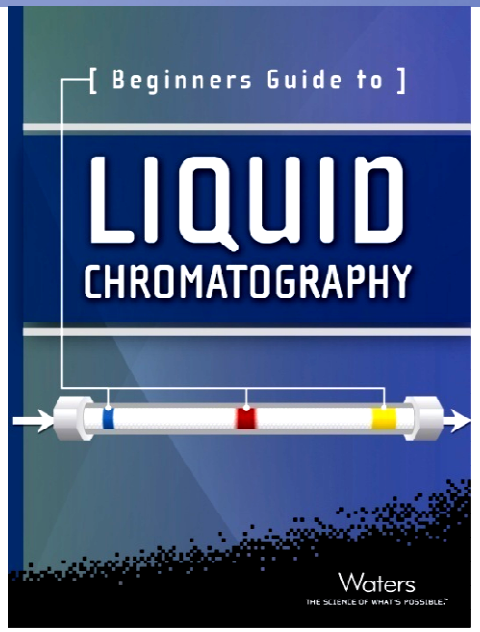

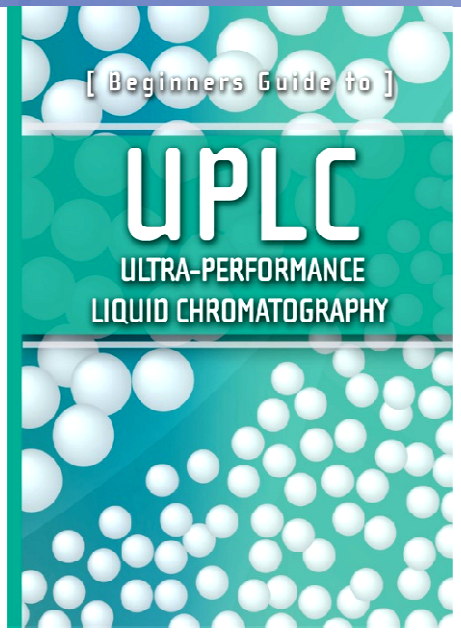

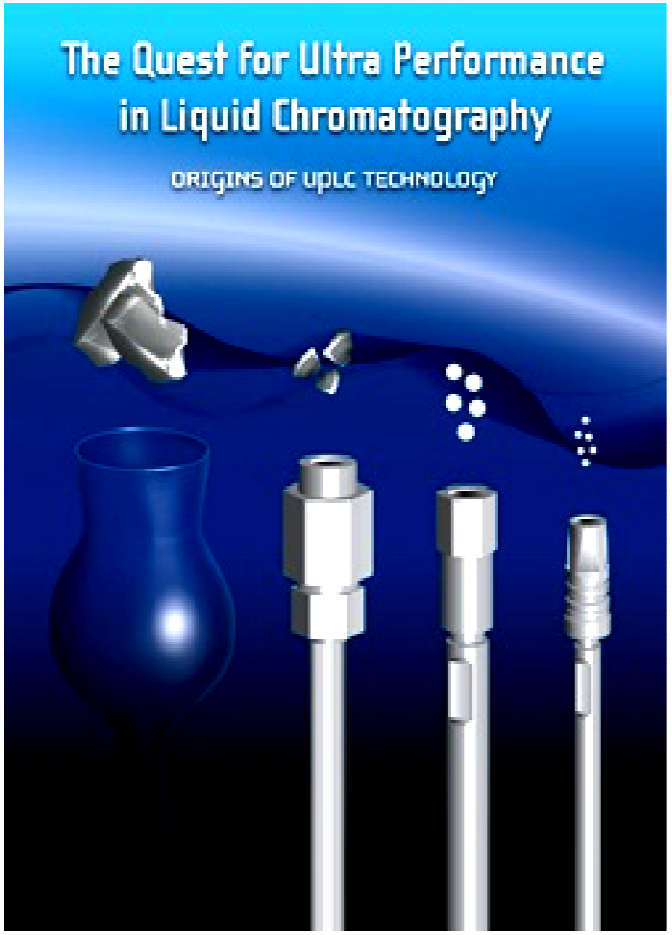

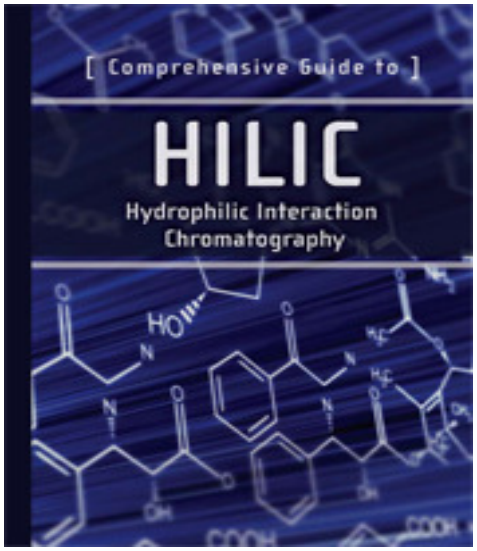

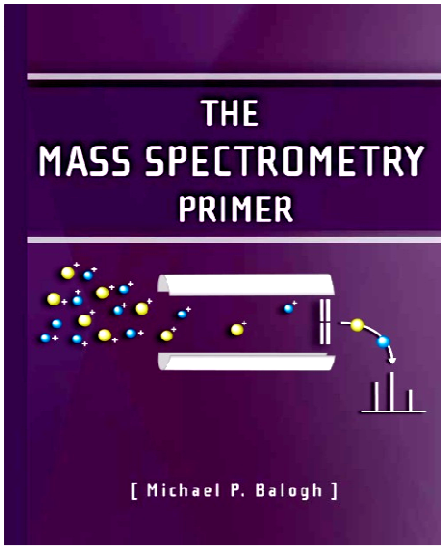

Closing

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Thank you for attending!