Introduction to Liquid Chromatography

Columns

System Components

Applications

Troubleshooting

Susan M. Steinike, M.S
HPLC Marketing Department
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A Brief History of Chromatography

- 1903: Russian botanist Mikhail Tswett separates plant pigments
- 1938: Russian scientists Izmailov and Shraiber use "drop chromatography", later perfected as Thin Layer Chromatography (TLC) by Kirchner in the U.S.
- 1952: Martin and Synge receive Nobel Prize for "invention of partition chromatography" or plate theory to describe column efficiency
- 1966: HPLC was first named by Horvath at Yale University but HPLC didn't "catch on" until the 1970s
- 1978: W.C. Stills introduced "flash chromatography", where solvent is forced through a packed column with positive pressure

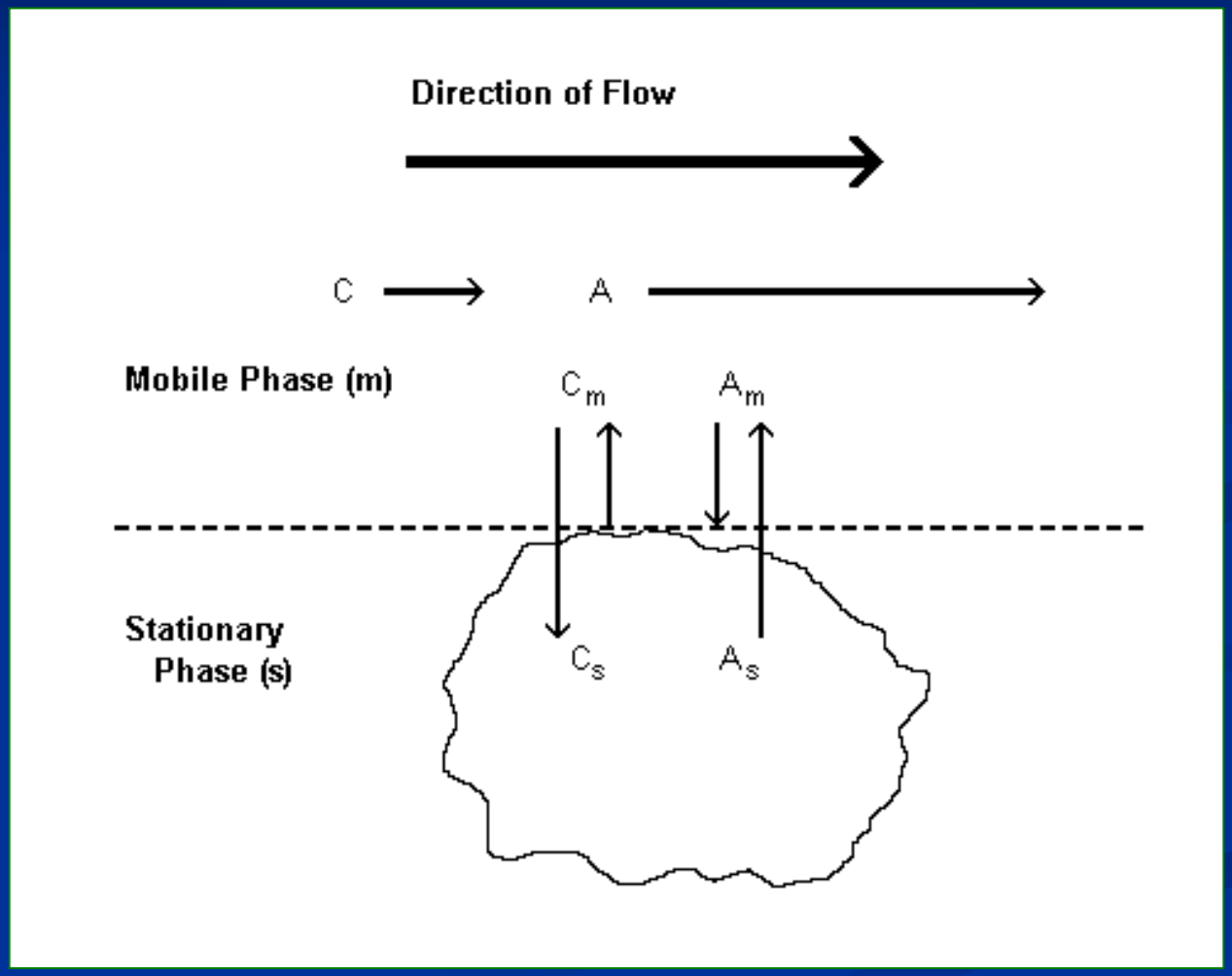
Modern HPLC

- Late 1970s/early 1980s
  - Instrumentation developed for high pressure solvent delivery: pumps, autosamplers, diode array detectors
  - More uniform packing material produced for columns
- Last 20 years
  - Nothing really "new", but by returning to the basic theory of chromatography, even better columns are on the market: smaller particle sizes which yield faster separations, but require hardware to withstand higher pressures.

What is Chromatography?

- Separation of a mixture into individual components.
- The separation uses a Column (stationary phase) and Solvent (mobile phase).
- The components are separated from each other based on differences in affinity for the mobile or stationary phase.
- The goal of the separation is to have the best RESOLUTION possible between components.

The Most Basic Explanation of Chromatography Ever



How Do You Get Separation?

- Hardware: pumps, injector, detector
- Column: particle diameter, column size, packing materials, and the dreaded equations
- Our seminar will focus on the contribution of each factor to perform separations.

Outline

- Column Considerations
  - Theory (including, well...you know)
  - Different Stationary Phases
- Hardware Components
  - Pumps, Injectors, Detectors, etc.
  - Examples of Application-Specific Configurations
- Applications
  - Pharmaceuticals and Proteomics
  - Food and Beverage, Environmental
  - Research and Method Development

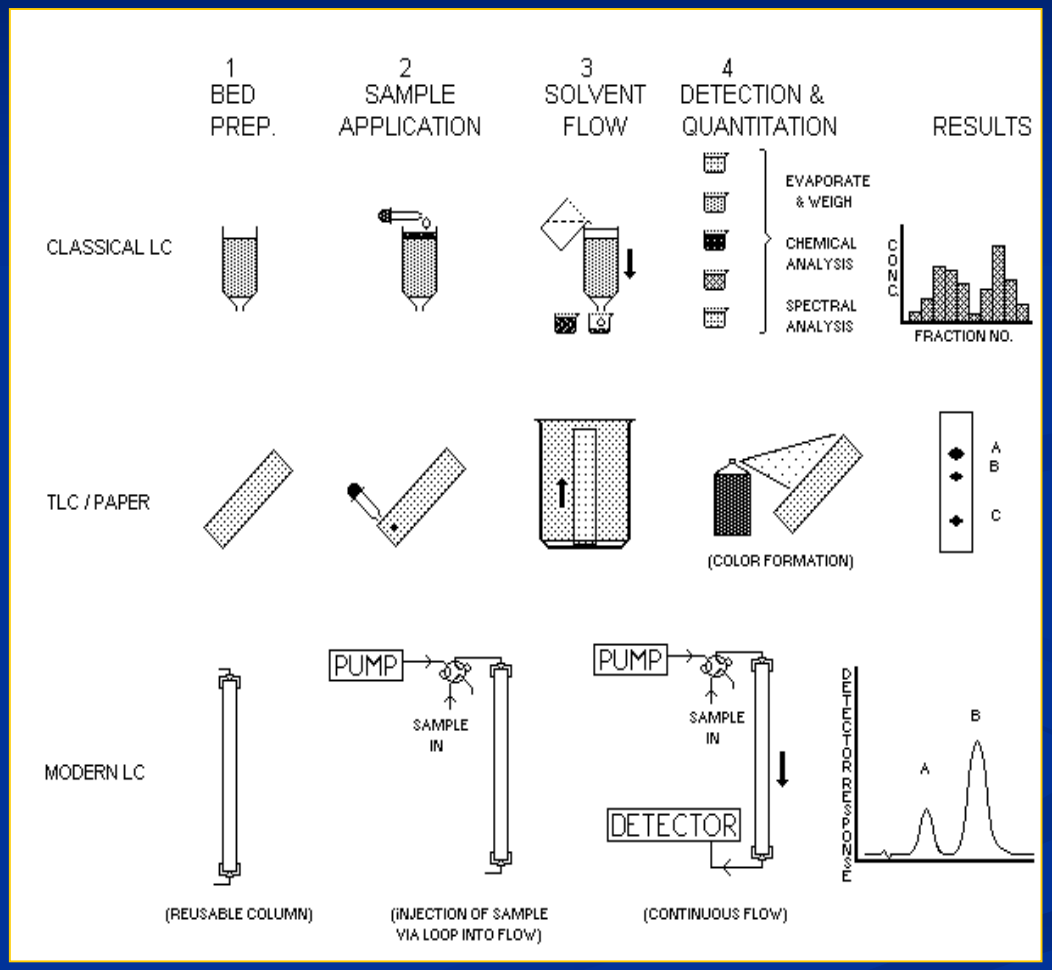
Outline

- System Troubleshooting
  - Leaks, Reproducibility, Column Care, and More
- Chromatography Software
  - Method and Sequence Setup
  - Calibration Curves and Reporting
- Chromatography Hardware
  - Modular LC-20 Prominence
  - Integrated LC-2010HT

Modern HPLC vs. Traditional LC Methods

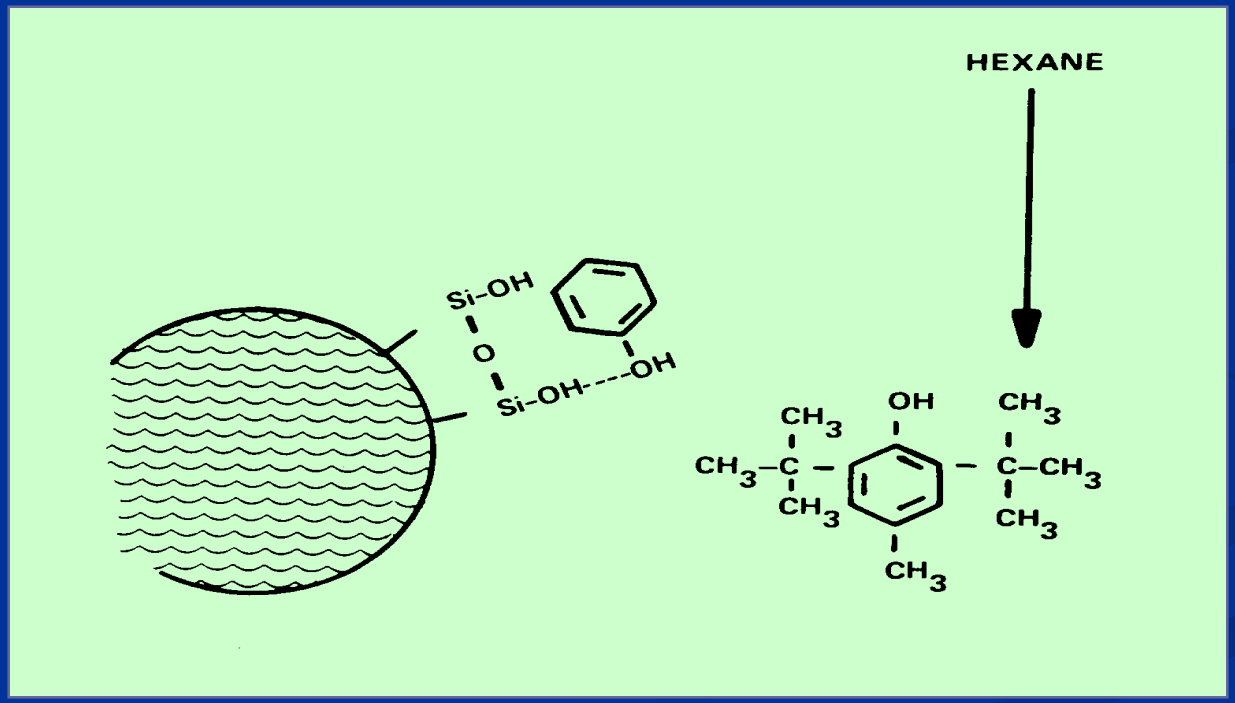
- Classical open-column LC.
- Thin-Layer Chromatography (TLC) and paper chromatography.
- In modern HPLC the columns and packings are, in general, highly refined, high in resolving capacity, and are reusable.

HPLC and Pre-HPLC Techniques



Column Types

- Normal Phase LC
  - Polar stationary phase: Silica
  - Nonpolar mobile phase: Hexane, Ethyl acetate
  - The LEAST polar compound comes out first



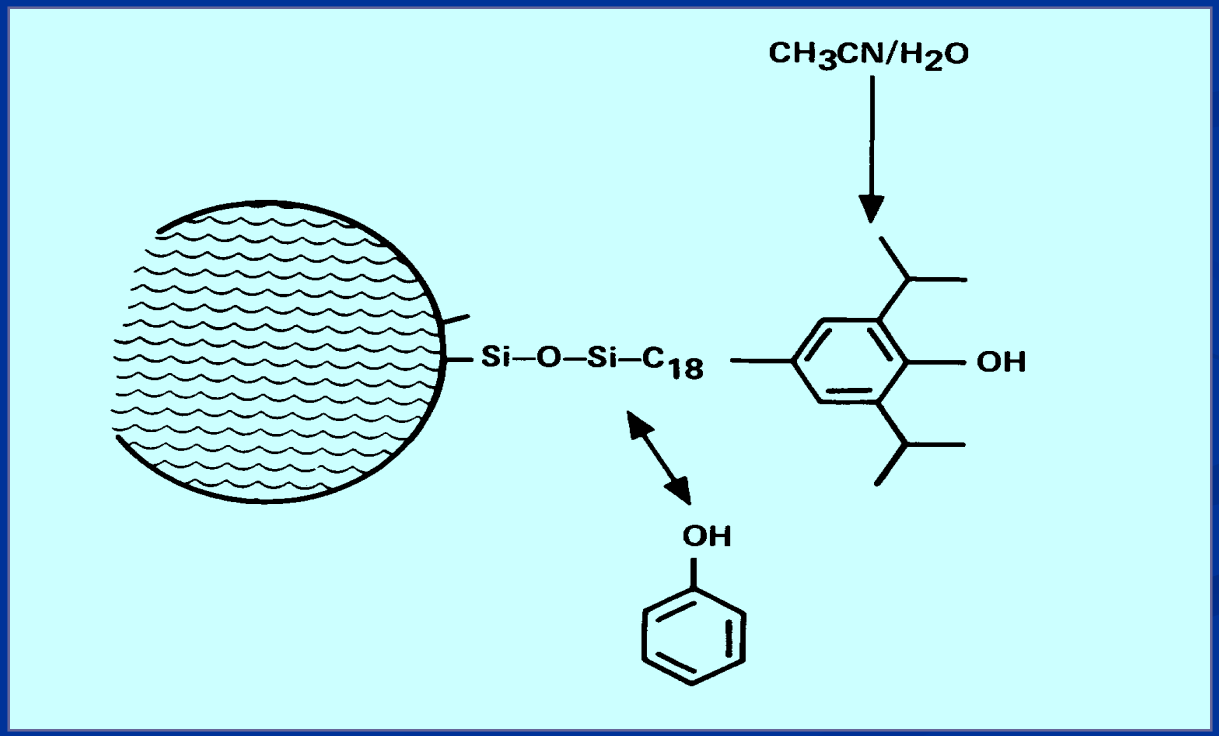
Normal Phase HPLC Columns

- Cyano: Rugged, moderate polarity, general use
- -OH (Diol): More polar and retentive
- Amino: Highly polar, less stable
- Silica: Very rugged, low cost, adsorbent (Unbonded)

The cyano column with a low polarity mobile phase (hydrocarbon with a amall amount of another solvent) will act as a normal phase column.

Column Types

- Reversed-Phase LC
  - Nonpolar stationary phase: C8, C18
  - Polar mobile phase: Water, ACN, Methanol
  - The MOST polar compound comes out first



Reversed Phase HPLC Columns

- C-18, C-8: Rugged, general purpose, highly retentive
- C-3, C-4: Less retentive, used mostly for peptides & proteins
- Phenyl: Greater selectivity than alkyl-bonded
- Cyano: Moderate retention, normal & rev. phase
- Amino: Weak retention, good for carbohydrates

The cyano column with a high polarity mobile phase (Water/MeOH) will act as a reversed phase column.

Normal vs. Reversed Phase

Parameter	Normal Phase	Reverse Phase
Polarity of Packing	Medium to High	Low to Medium
Polarity of Solvent	Low to Medium	Medium to High
Elution Sequence	Low Polarity First	High Polarity First
Increase Solvent Polarity	Faster Elution	Slower Elution

Column Types

- Ion Exchange LC
  - Stationary phase contains charged groups
  - SAX (Strong Anion Exchange): NH_3^+
  - WAX (Weak Anion Exchange): NR_2H^+ (DEAE)
  - SCX (Strong Cation Exchange): SO_3^-
  - WCX (Weak Cation Exchange): Carboxymethyl (CM)
  - More highly charged analytes have stronger retention
  - More "bulky" stationary phases have weaker retention

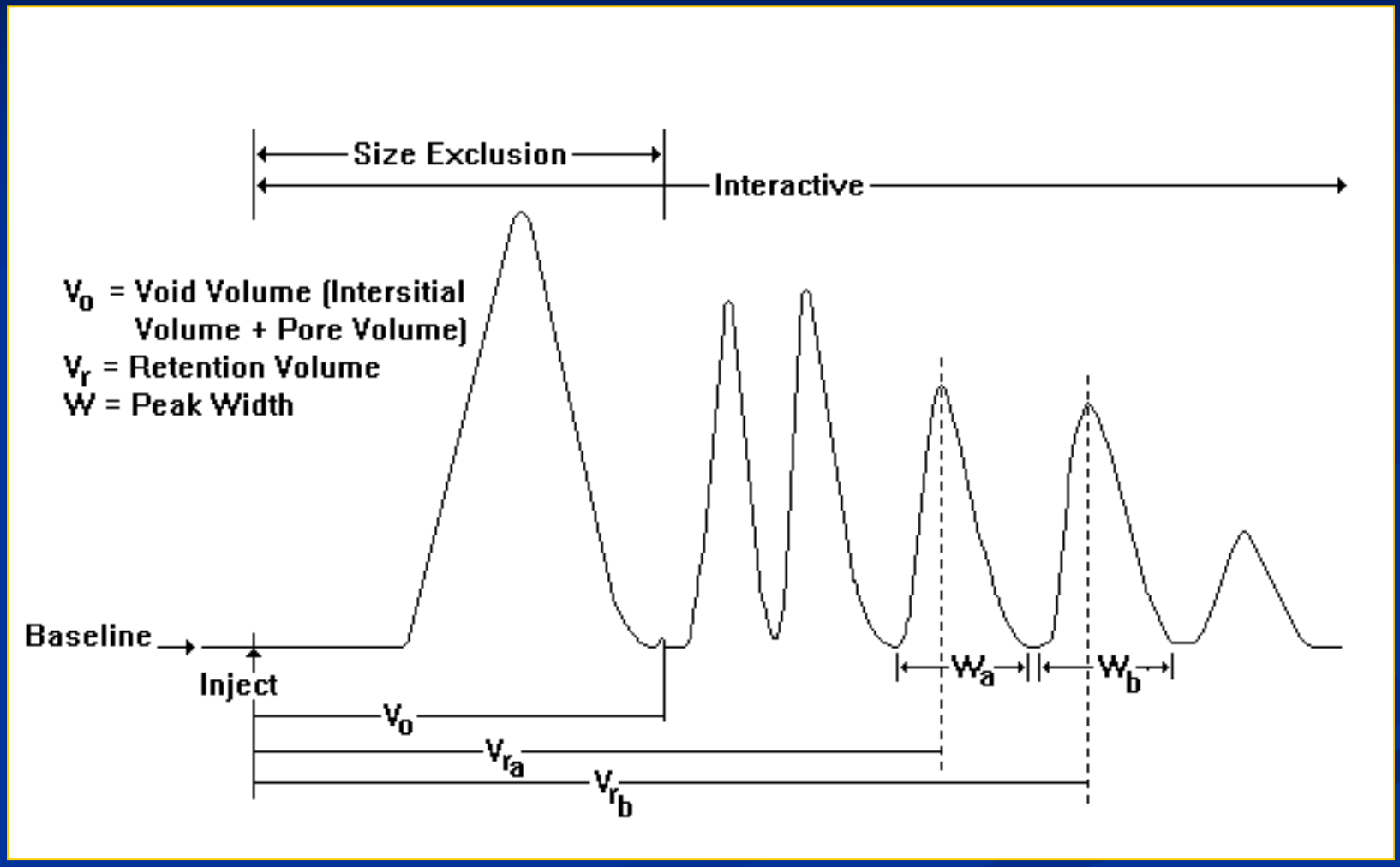
Column Types

- Size Exclusion LC (also called Gel Permeation)
  - Stationary phase is a polymer (polystyrene-divinyl benzene or acrylamide) with a defined pore size
  - Large compounds cannot fit into the pores and elute first
  - Used to determine molecular weight distribution of polymers

Typical Column Sizes

- Particle size: 5 μm, 3 μm, and smaller
  - Monodispersed means particles are the same size
  - Very important for stable pressure and flow
  - Smaller particles produce higher system pressure
- Pore size: 100-120 A is typical
- Surface area: 300-350 m^2/g
- Carbon load: 9-12% for C8, 16-20% for C18
  - Higher carbon load = better resolution but longer run times
  - Lower carbon load = shorter run times, but may change selectivity vs. higher carbon load

Idealized HPLC Separation



Void Volume

- The void volume is the amount of "dead" volume in the column that is not taken up by the particles of stationary phase.
- In general, there is approximately 0.1 mL of void volume for each cm of column length, for columns with a 4.6 mm i.d. and 5 µm particles

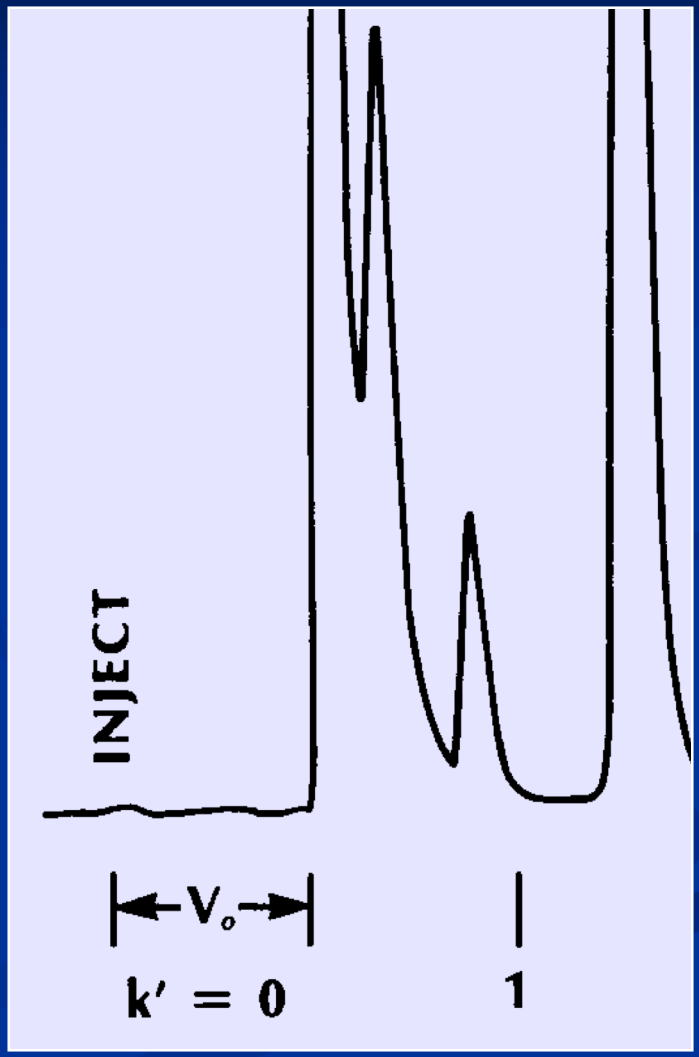
$$V_m \approx 0.5 d_c^2 L$$

Where V_m is the column volume in mL,
L is the column length in cm, and
d_c is the inner diameter in cm

Void Volume

- The void volume is exactly determined by injecting a compound that is completely unretained, then using the chromatogram to calculate void volume.

- Elution time x flow rate = void volume



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Factors Influencing Resolution

Capacity Factor, k'
Selectivity Factor, α
Efficiency, N

The Resolution Equation

- Resolution is defined as the completeness of separation from one analyte to another
- In general, resolution may be expressed as:

$$Rs = 2(Vrb - Vra)/(Wa + Wb)$$

$$= 2(trb - tra)/ (Wa + Wb)$$

- Where Vra/b = retention volume of peak a/b
- Where tra/b = retention time of peak a/b
- Where Wa/b = width of peak a/b

Resolution

- For closely eluting or adjacent peaks, the resolution equation may be expressed as:

$$R_s = 1/4[(\alpha - 1)/\alpha]\sqrt{N}[k'/(1+k')]$$

- The terms of capacity factor (k'), selectivity (α), and efficiency (N) all contribute to resolution
- Let's look at how each term affects resolution

Capacity Factor, k'

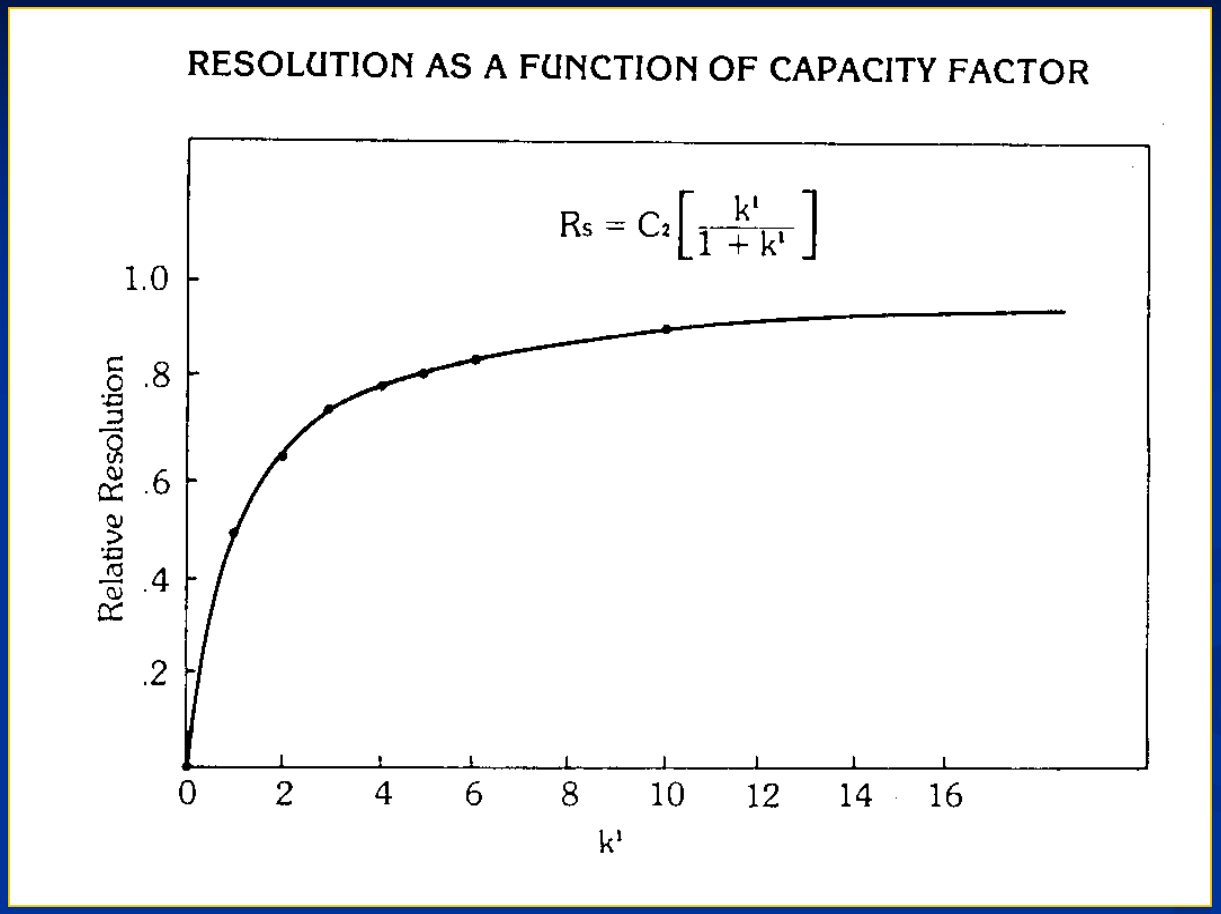
The relative degree to which an analyte component is delayed as it is eluted through a given system (retentivity).

$$k' = (V_r - V_0)/V_0 = (t_r - t_0)/t_0$$

Where V_r = peak retention volume; V_0 = column void volume
t_r = peak retention time; t_0 = peak void time

- The larger the k', the later the analyte elutes after the void.

Effect of k' on Overall Resolution



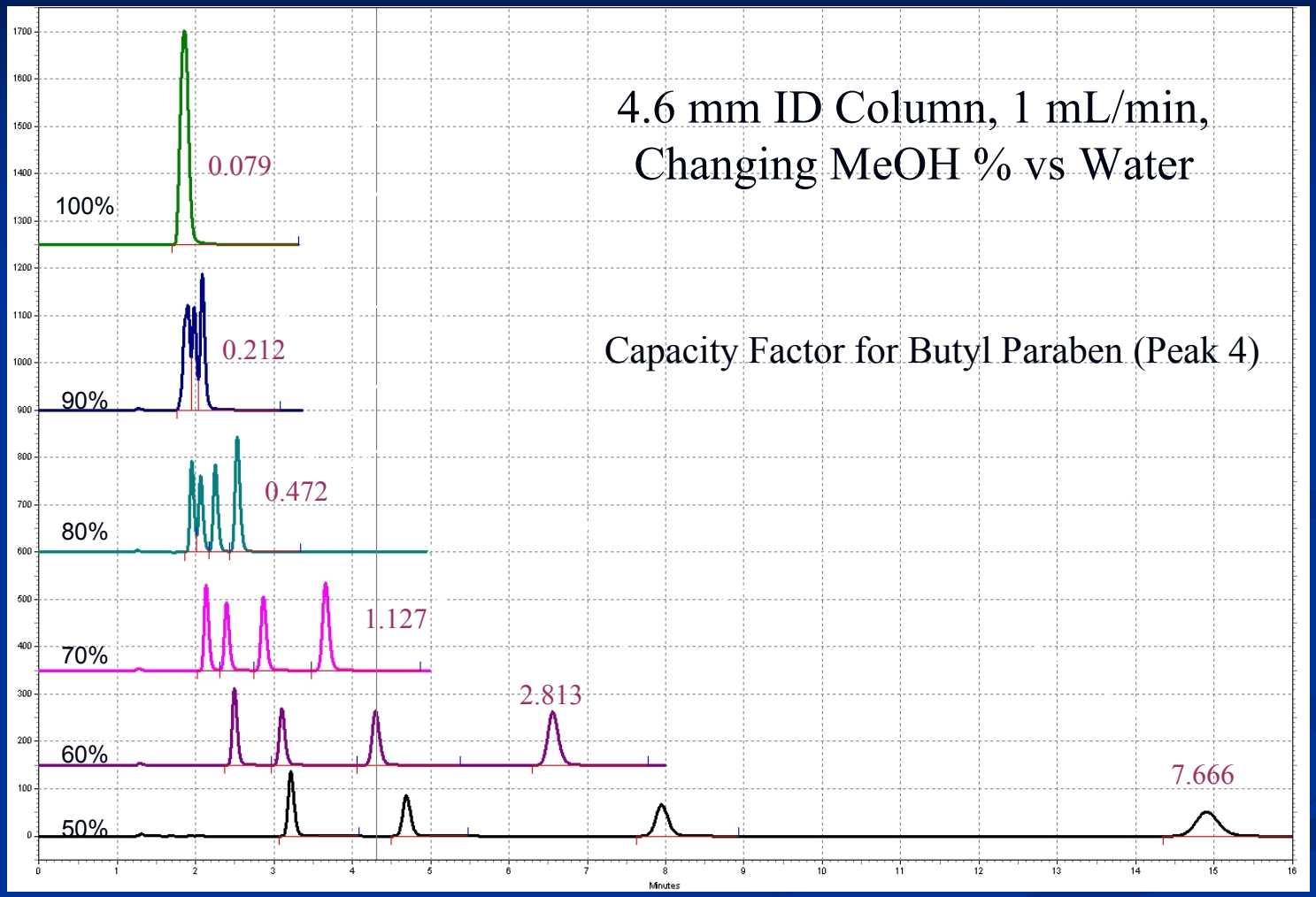
- As k' grows larger, its effect reaches a limit at a value of about 10.
- Since k' depends on retention time, longer columns eventually have a diminished effect on resolution.

Influencing the Capacity Factor k'

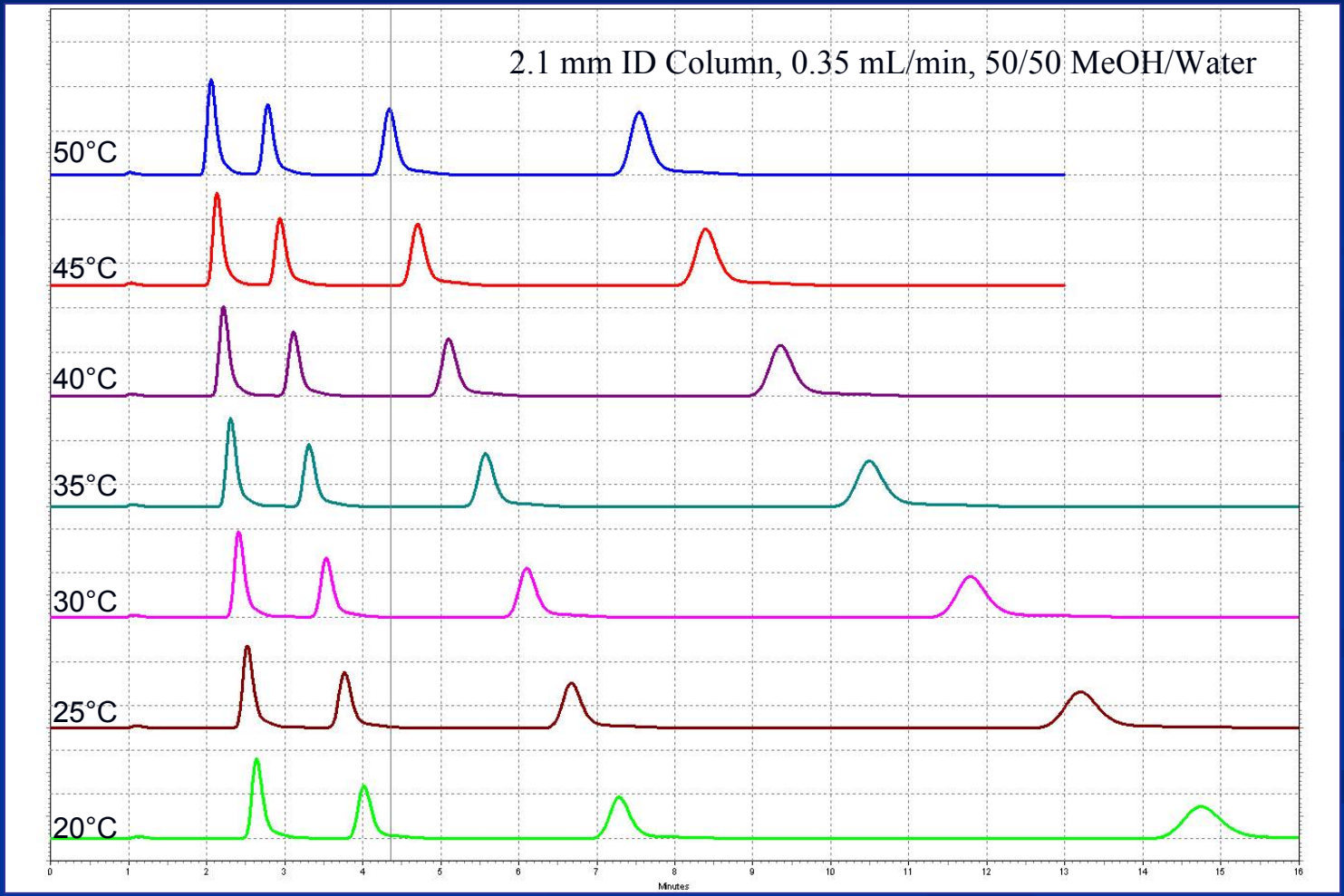
- Retentivity (k') decreases 2 - 3 fold for each 10% increase in mobile phase strength.
- Mobile Phase Strength -
  - As per the rule of thumb, altering the mobile phase strength also alters the retention of the analytes.
- Bonded Phase Functionality (Reverse Phase) -
  - As the bonded phase hydrophobicity increases (increasing alkyl chain length, etc.) so will the retention of the analytes.
- Temperature -
  - As temperature increases, the retention time decreases. This does not necessarily result in poorer separation because of the other factors in the resolution equation.

Which of these is easiest to change??

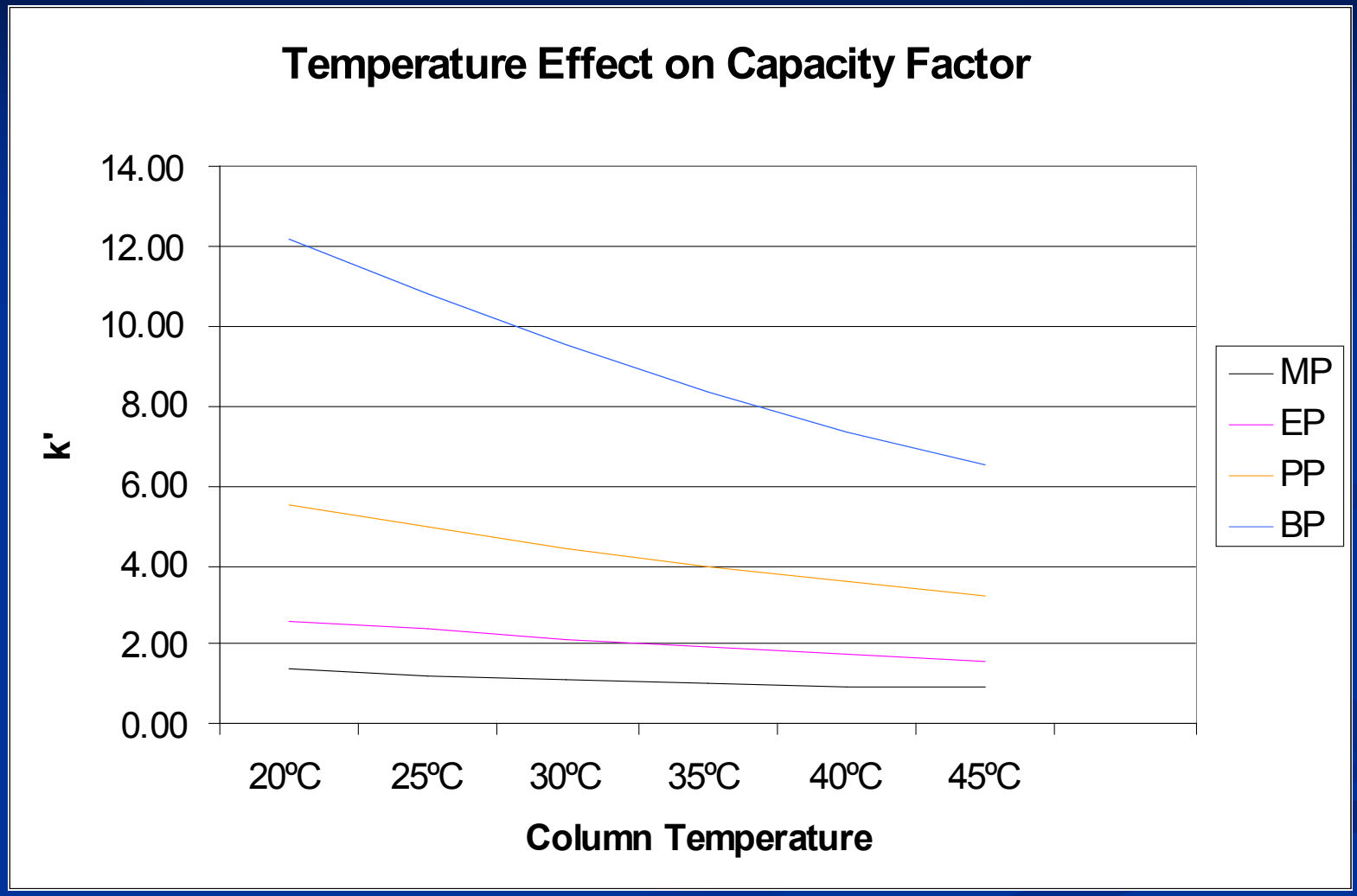
Mobile Phase Strength vs. k'



Temperature Effect on k'



Temperature Effect on k'



Summary of k' Effects

- A larger value of k' means better resolution...to a certain extent (k' = 10 maximum)
- Increasing the mobile phase strength decreases k'
- Increasing the temperature decreases k', but may not result in a "bad" separation based on the other factors affecting resolution

Selectivity Factor, α

- The selectivity or separation factor represents the ratio of any two adjacent k' values, thereby describing the relative separation of adjacent peaks. This relationship is expressed as:

$$\alpha = k'b/k'a$$

  - If α = 1, two components are perfectly overlapping
  - For early eluting peaks you want α to be large for good resolution.
  - For later eluting peaks, α can be smaller and still have acceptable separation.

Effect of α on Overall Resolution

- Remember the resolution equation?

$$R_s = 1/4[(\alpha - 1)/\alpha]\sqrt{N}[k'/(1+k')]$$

- Let's only look at the part involving α

$$R_s = 1/4[(\alpha - 1)/\alpha]$$

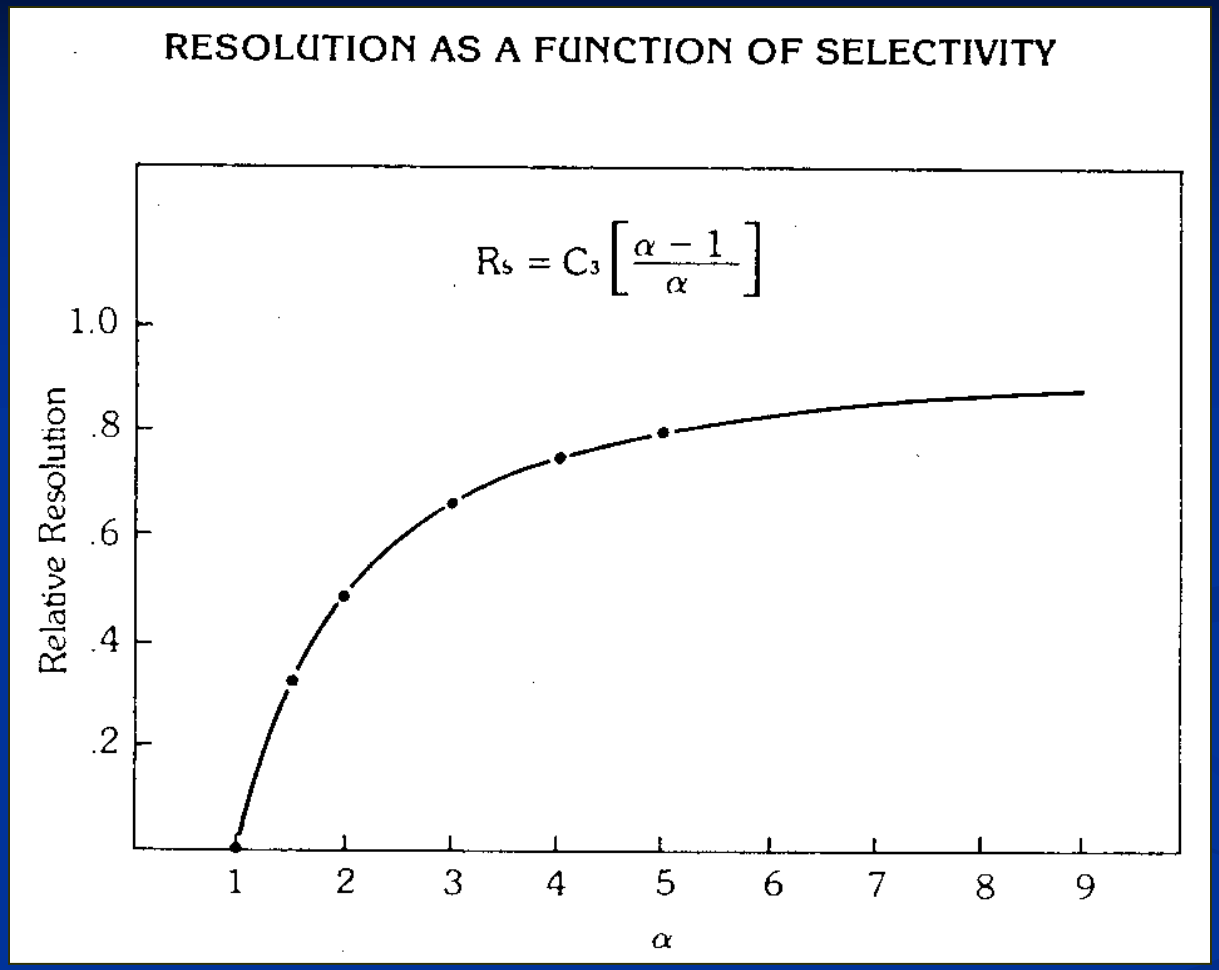
- And see how much resolution will improve with small changes in α

Effect of α on Overall Resolution

$$R_s = 1/4[(\alpha - 1)/\alpha]$$

- For an α value of 1.1, the contribution of the selectivity term is
  - (1.1 – 1) / 1.1 = 0.09
- For an α value of 1.4, the contribution of the selectivity term is
  - (1.4 – 1) / 1.4 = 0.29
- So...a very small change in α leads to a more than THREE-FOLD increase in the contribution to resolution.

Effect of α on Overall Resolution



- As α grows larger, its effect reaches a limit at a value of about 5.
- Since α depends on components' retention factor k', longer columns eventually have a diminished effect on resolution.

Influencing the Selectivity Factor α

- Mobile Phase Type -
  - The importance of the type of interactions between the mobile phase and analytes is critical to the optimization of the selectivity of a system.
- Column Type -
  - The bonded phase functionality can be selected by its chemical nature to provide better selectivity in an analytical method.
- Temperature -
  - Selective interactions between analyte molecules and the stationary phase may not become evident until a critical temperature is attained.

Which of these is easiest to change??

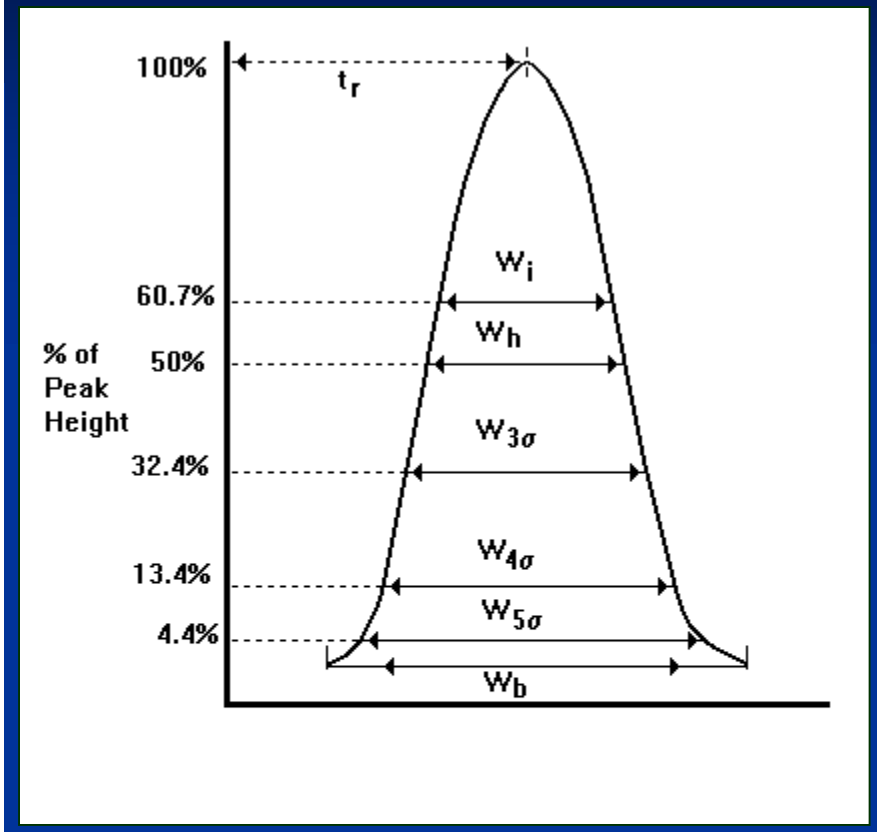
Summary of α Effects

- Since α is the ratio of two k' values, the same general statements apply:
  - Increasing the mobile phase strength decreases individual values of k', but their ratio (α) may affect resolution
  - Increasing the temperature decreases individual values of k', but their ratio (α) may significantly affect resolution.
- A small increase in α leads to a large increase in resolution

Column Efficiency, N

- The column efficiency is defined as the degree to which a column and/or other system components can physically and chemically affect the separation of analytes.
- As column efficiency increases, analyte components will elute in a smaller volume of the mobile phase, usually observed as narrower or "sharper" peak shapes.
- Column efficiency is generally expressed in terms of theoretical plate number.

Calculation of Theoretical Plates



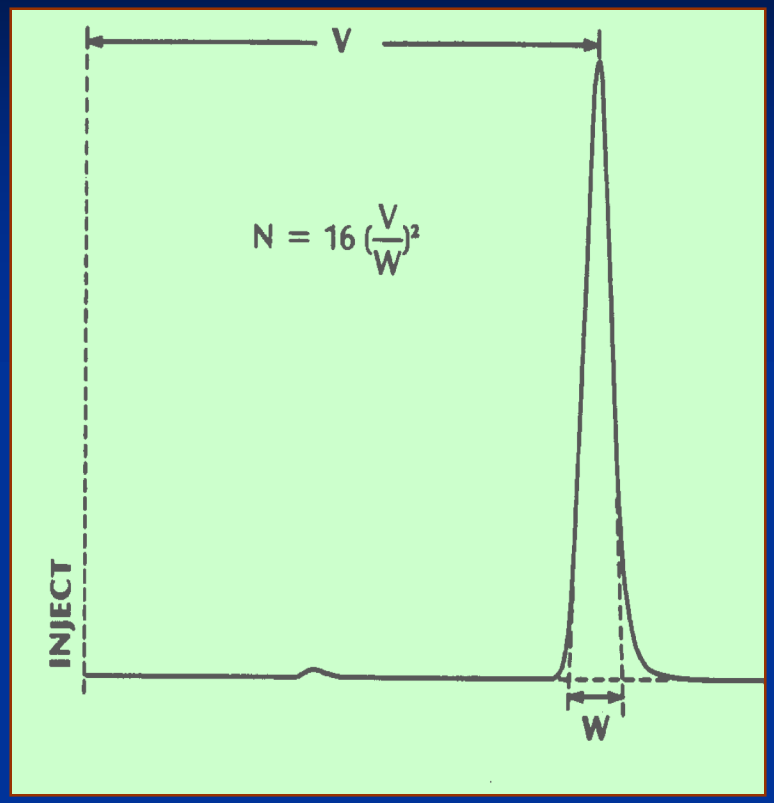
$$N = A(t_r / W)^2$$

W	A	Method	Width measured at
W_i	4	Inflection	Inflection point (60.7% of peak height)
W_h	5.54	½ Height	50% of peak height
$W_{3\sigma}$	9	3σ	32.4% of peak height
$W_{4\sigma}$	16	4σ	13.4% of peak height
$W_{5\sigma}$	25	5σ	4.4% of peak height
W_b	16	Tangent	Baseline, following tangent drawing

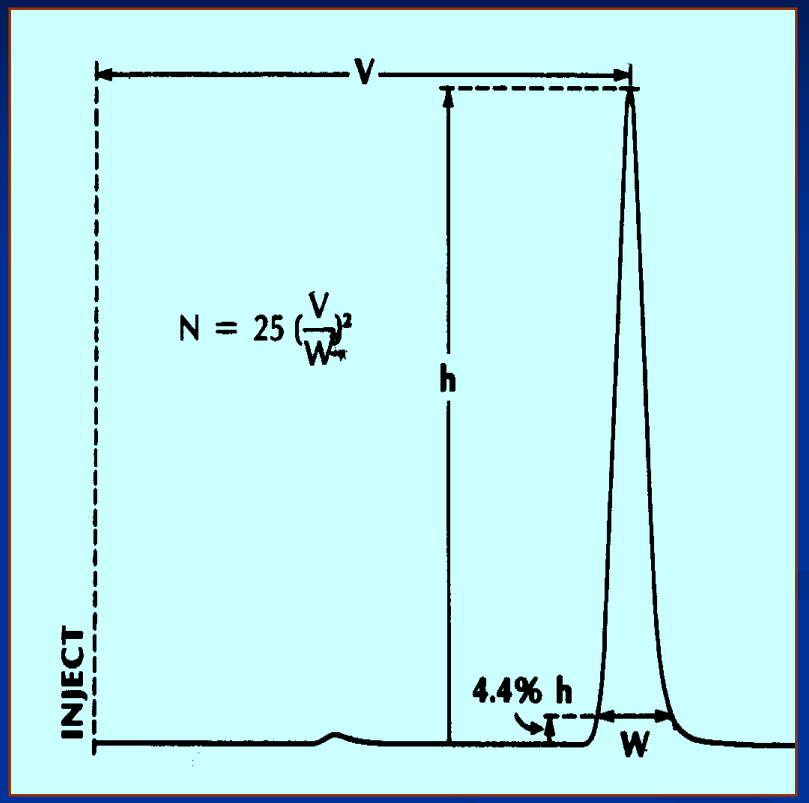
Constants A are different at each peak width, assuming a perfect Gaussian shape.

Real-world peaks often have tailing, so widths measured at the lower part of the peak more accurately reflect the tailing when calculating N.

Calculation of Efficiency, N



- Width measured at the baseline after tangent lines are drawn on the peak. Used when tailing is minimal.



- Width measured at 4.4% of peak height, no tangents drawn. Used when tailing is significant.

Effect of N on Overall Resolution

- Do you STILL remember the resolution equation?

$$R_s = 1/4[(\alpha - 1)/\alpha]\sqrt{N}[k'/(1+k')]$$

- Now let's look at the part involving N

$$R_s = 1/4\sqrt{N}$$

- And see how much resolution will improve with changes in N

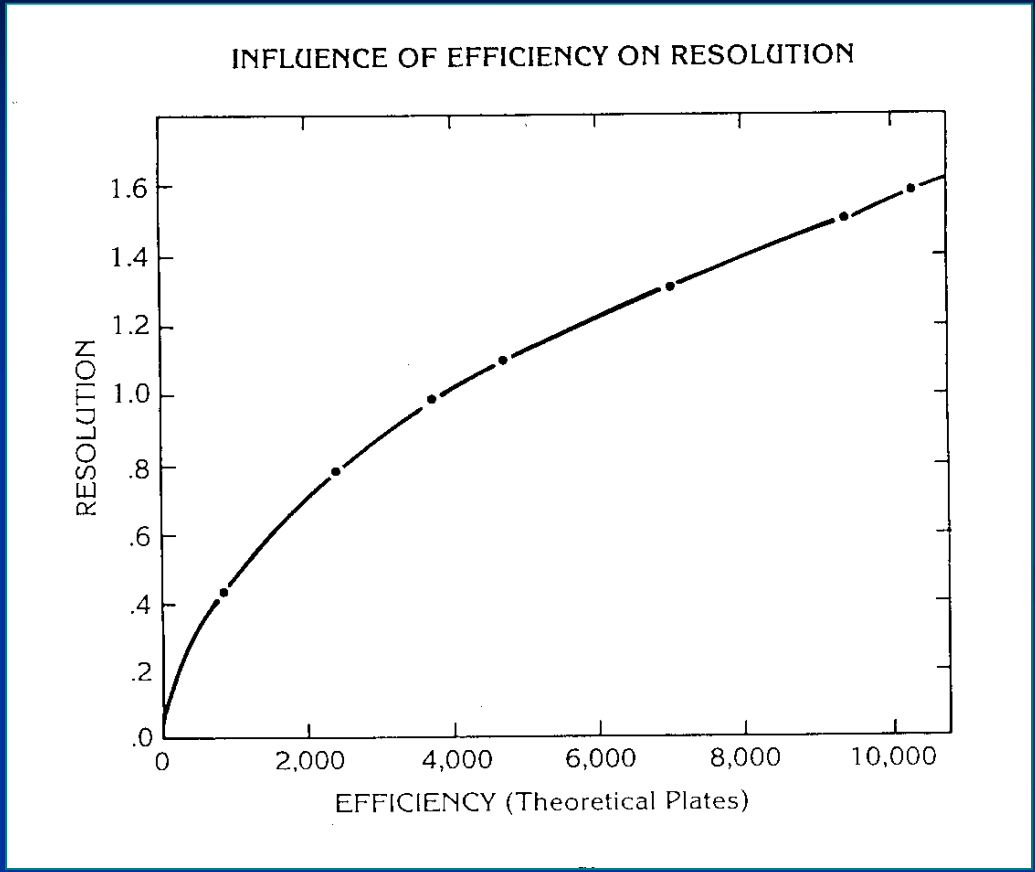
Effect of N on Overall Resolution

$$R_s = 1/4\sqrt{N}$$

Plates	√N	Contribution
5,000	70.7	- - - -
10,000	100	41%
20,000	141.4	100%

- Since the contribution of N to resolution is a square root, doubling N from 5000 to 10,000 only increases the contribution to resolution by 41%.
- To double the effect on resolution coming from N, we have to increase the value of N by a factor of 4

Effect of N on Overall Resolution



- Note that there is no flattening of the curve like with k' and α.
- Resolution will continue to increase as theoretical plates increase.

Influencing the Efficiency, N

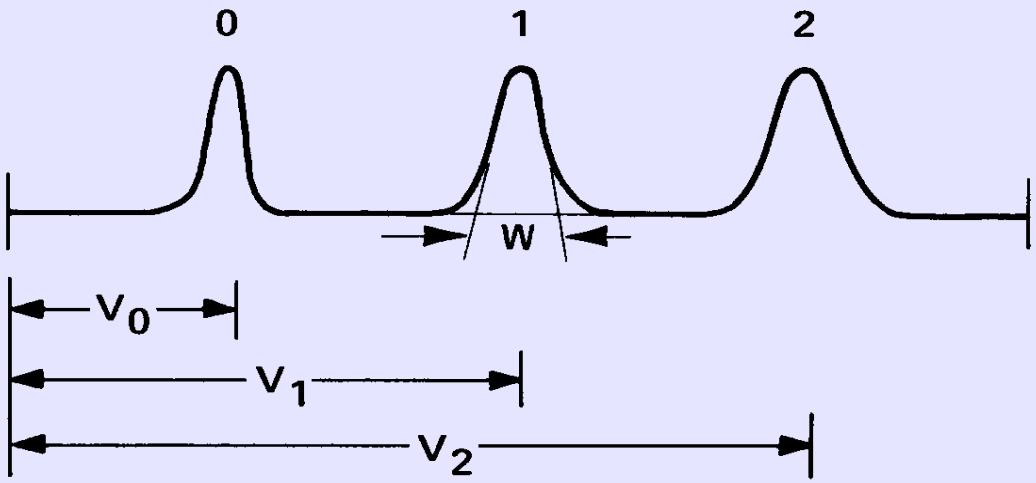
- Particle Size and Size Distribution -
  - The smaller the particle size and the narrower the range of the particle size distribution, the more efficient the column.
- Packing Type -
  - Totally porous particles will also have greater efficiency than solid or pellicular-shaped packings, due to the additional surface area attributable to the pores.
- Mobile Phase Viscosity -
  - As mobile phase viscosity increases, molecular movement through the mobile phase is inhibited.
- Temperature -
  - For reverse phase chromatography, an increase in efficiency, N, may be realized as column temperature is increased.

Effect of Particle Size on N

Column Diameter (mm)	Column Length (cm)	Particle Size (µm)	4σ Peak Width (µL)	Theoretical Plates per centimeter
10	25	10	1118	333
4.6	25	10	237	333
4.6	25	5	167	667
4.6	10	5	106	667
4.6	10	3	82	1111
4.6	3	3	45	1111
3	10	5	45	667
2	25	10	45	333
2	25	5	32	667
2	10	5	20	667
2	10	3	15	1111
1	25	10	11	333
1	25	5	8	667
1	25	3	6	1111
1	10	5	5	667
1	10	3	4	1111

Smaller particle sizes result in higher numbers of theoretical plates

Summary: Review of Terms



RETENTION , $k'_1 = \dfrac{V_1 - V_0}{V_0}$

SELECTIVITY, $\alpha = \dfrac{k'_2}{k'_1} = \dfrac{V_2 - V_0}{V_1 - V_0}$

PLATES , $N = 16 \left(\dfrac{V_1}{W_1} \right)^2$

Summary: Relative Influence of All Factors on Resolution

Parameter Change	N	k'	α	R_s
Standard	10,000	2	1.1	1.52
+10% N	11,000	2	1.1	1.59
-25% N	7,500	2	1.1	1.31
-50% N	5,000	2	1.1	1.07
-60% N	4,000	2	1.1	0.96
-75% N	2,500	2	1.1	0.76
+10% k'	10,000	2.2	1.1	1.56
+10% α	10,000	2	1.2	2.78

Note that changing α a very small amount has the biggest effect

Summary: Review of Factors

PARAMETER	INFLUENCED BY	TARGET VALUE
Efficiency, N	Column, System Flowpath Configuration	Minimum of 400 Theoretical Plates per centimeter
Capacity Factor, k'	Mobile Phase Strength	1.0 - 10
Selectivity, α	Mobile Phase Type, Stationary Phase Type	1.1 - 2
Resolution, R_s	All of the Above	1.3 - 1.5 or Greater

Questions About Columns?

Next – HPLC System Components

HPLC System Components

- Pumps
  - Micro to Analytical to Preparative Flow Rates
  - Isocratic and Gradient Configurations
- Degasser
  - How it Affects Pumping and Sample Injection
- Valves
  - Solvent Selection and Flow Selection

HPLC System Components

- Sample Injection
  - Manual Injector or Autosampler
- Oven
  - How Temperature Affects Separation
  - Valves for Column Switching
- Detectors
  - UV-VIS
  - Diode Array
  - Fluorescence
  - Light Scattering
  - Refractive Index
  - Conductivity
  - Mass Spectrometer

HPLC System Components

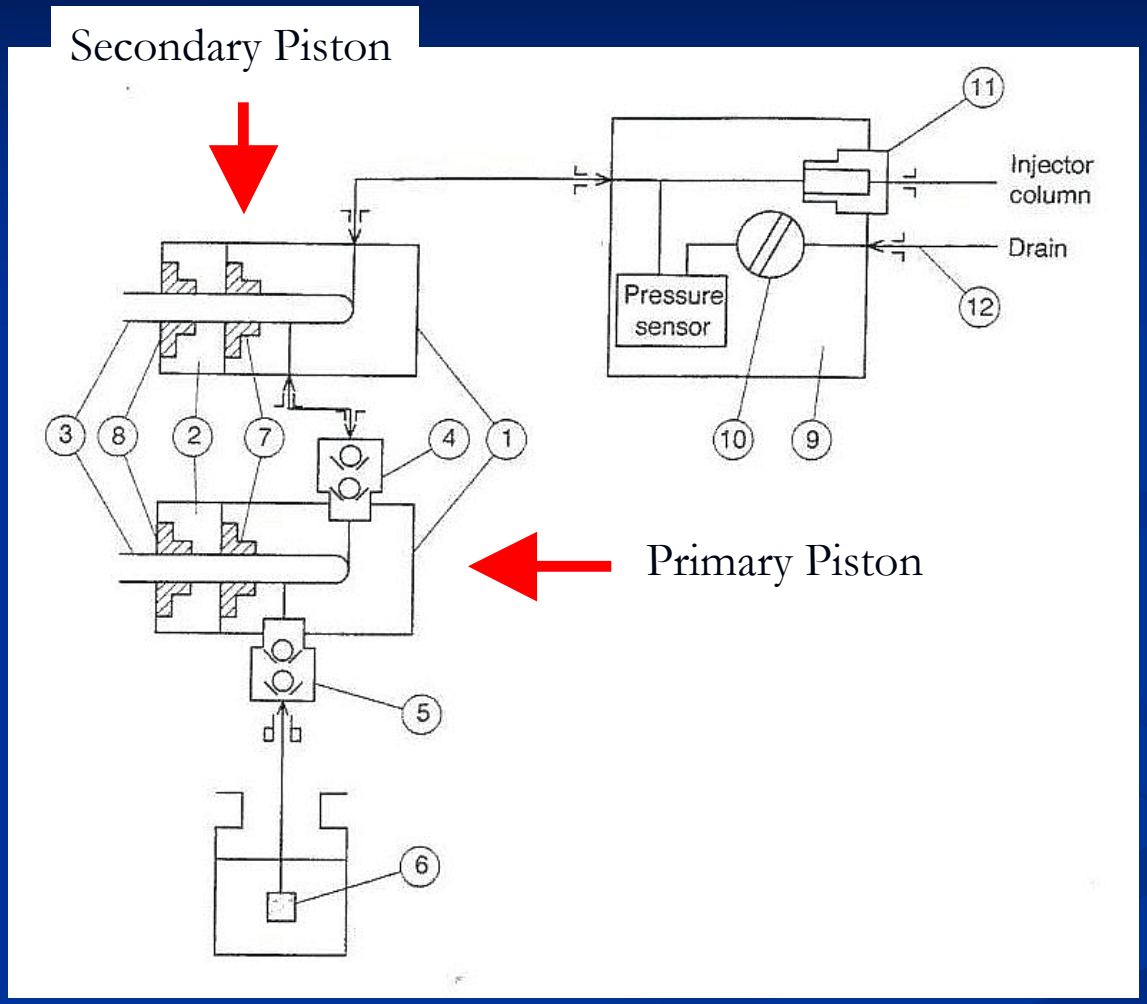
- Fraction Collector
  - Isolate Specific Sample Components
  - Purify Compounds for Multi-Step Synthesis
- Column
  - Types of Packing Material
  - Factors Affecting Separation
    - Particle Size and Column Length
    - Flow Rate and Temperature

Hardware Components of an HPLC System

HPLC Pumps – 2 Basic Types

- Tandem piston
  - Two pistons with different volumes (48 and 24 µL)
  - During each stroke, 24 µL of liquid is delivered
  - Best for higher analytical flow rates, up to 10 mL/min
  - Some pulsation is observed, and pulse dampeners are available
  - Not recommended for pulse-sensitive detectors like RID and CDD

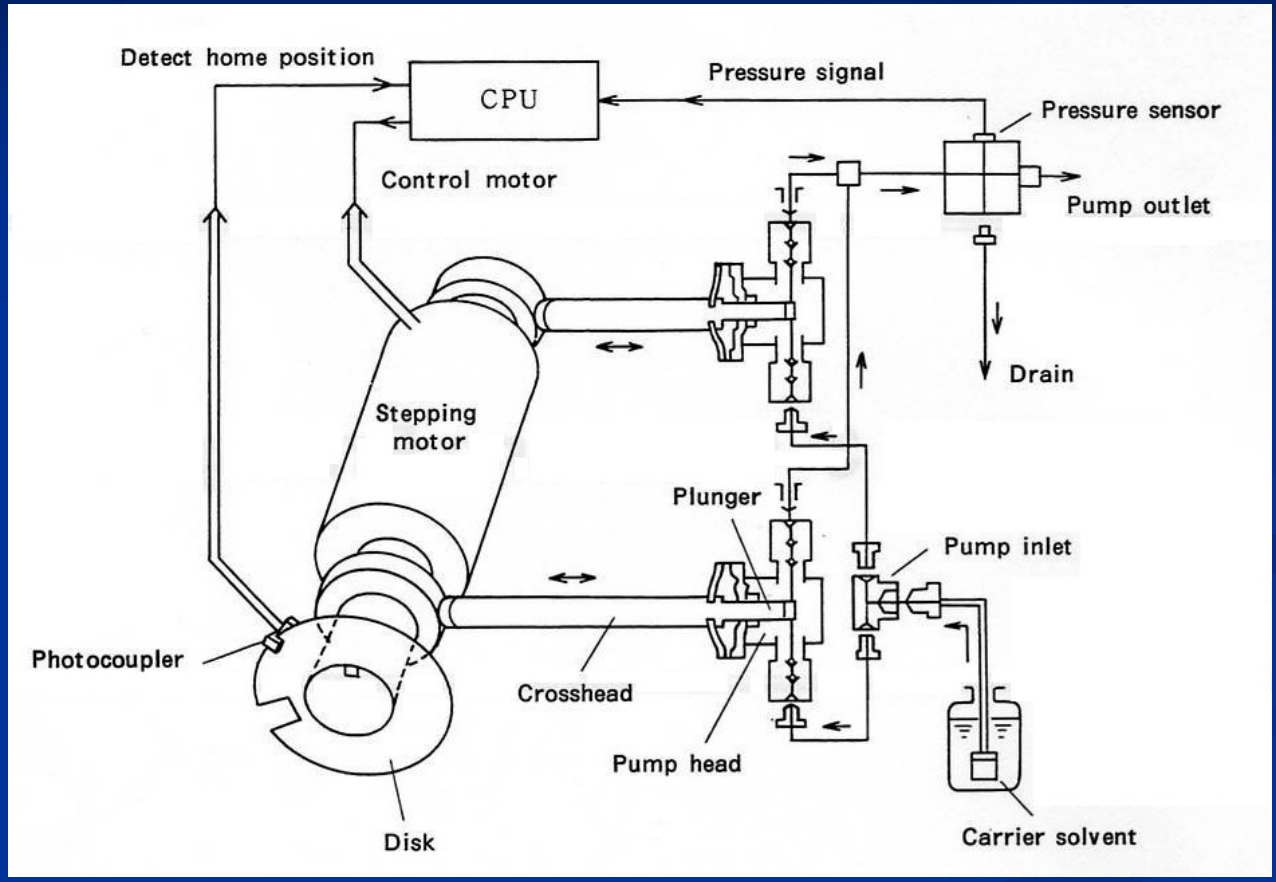
Tandem Piston Pump



HPLC Pumps – 2 Basic Types

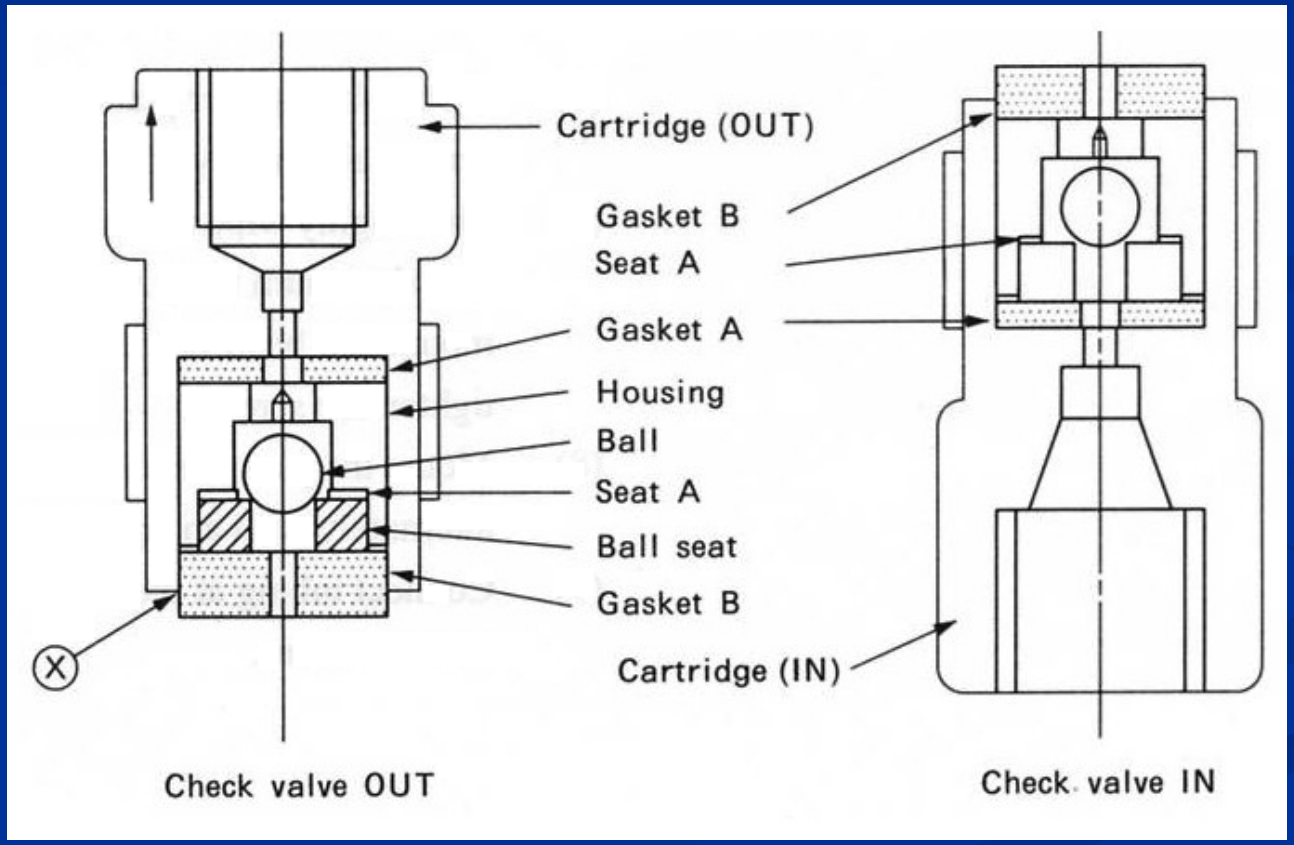
- Dual Piston
  - Two pistons with equal volume (10 µL each)
  - During each stroke, 10 µL is delivered
  - Best for low flow rates (< 1 mL/min)
  - Little to NO pulsation, so it's ideal for pulse sensitive detectors like RID and CDD

Dual Piston Pump



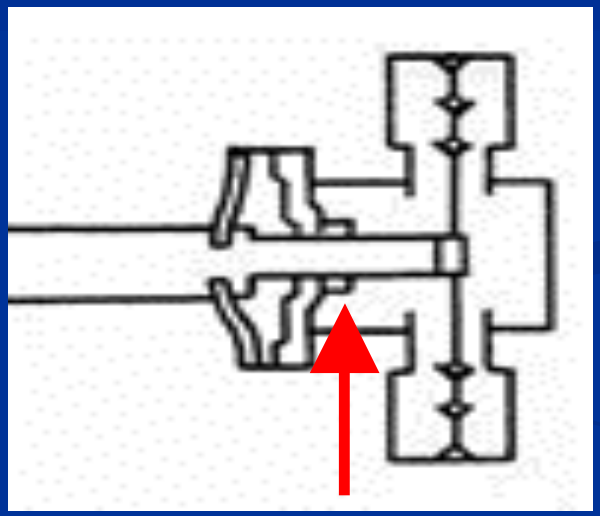
Other Pump Components

- Check Valves
  - Control liquid movement in and out of the pump head



Other Pump Components

- Piston/plunger seal
  - Prevents solvent leakage out of pump head



Seal

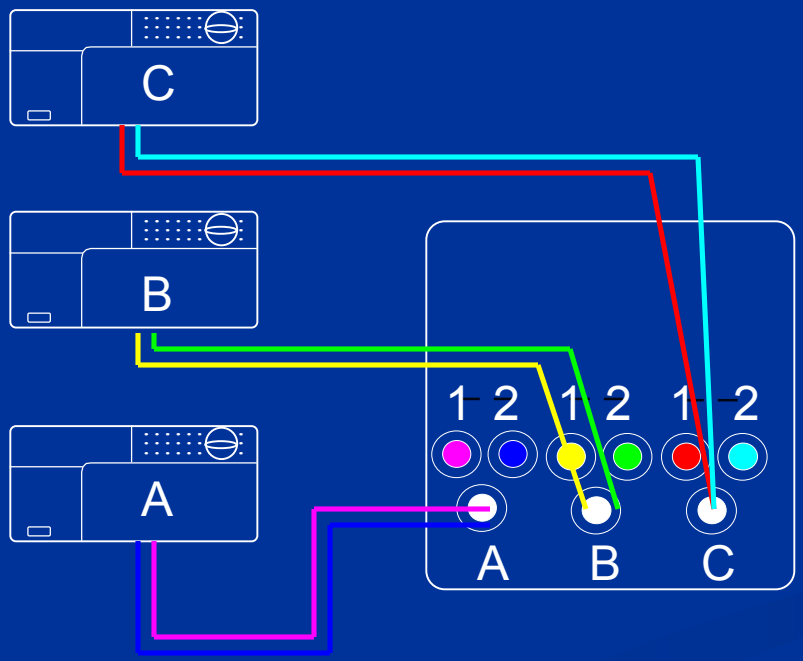
- Inline filter
  - Removes solvent particulates

HPLC Degassing

- Degassing removes dissolved air that interferes with check valve operation
- Helium sparge
  - Gas line from the tank directly in the solvent bottle
- Vacuum degassing
  - Sonicate before connecting to the system
  - Online with a degassing unit

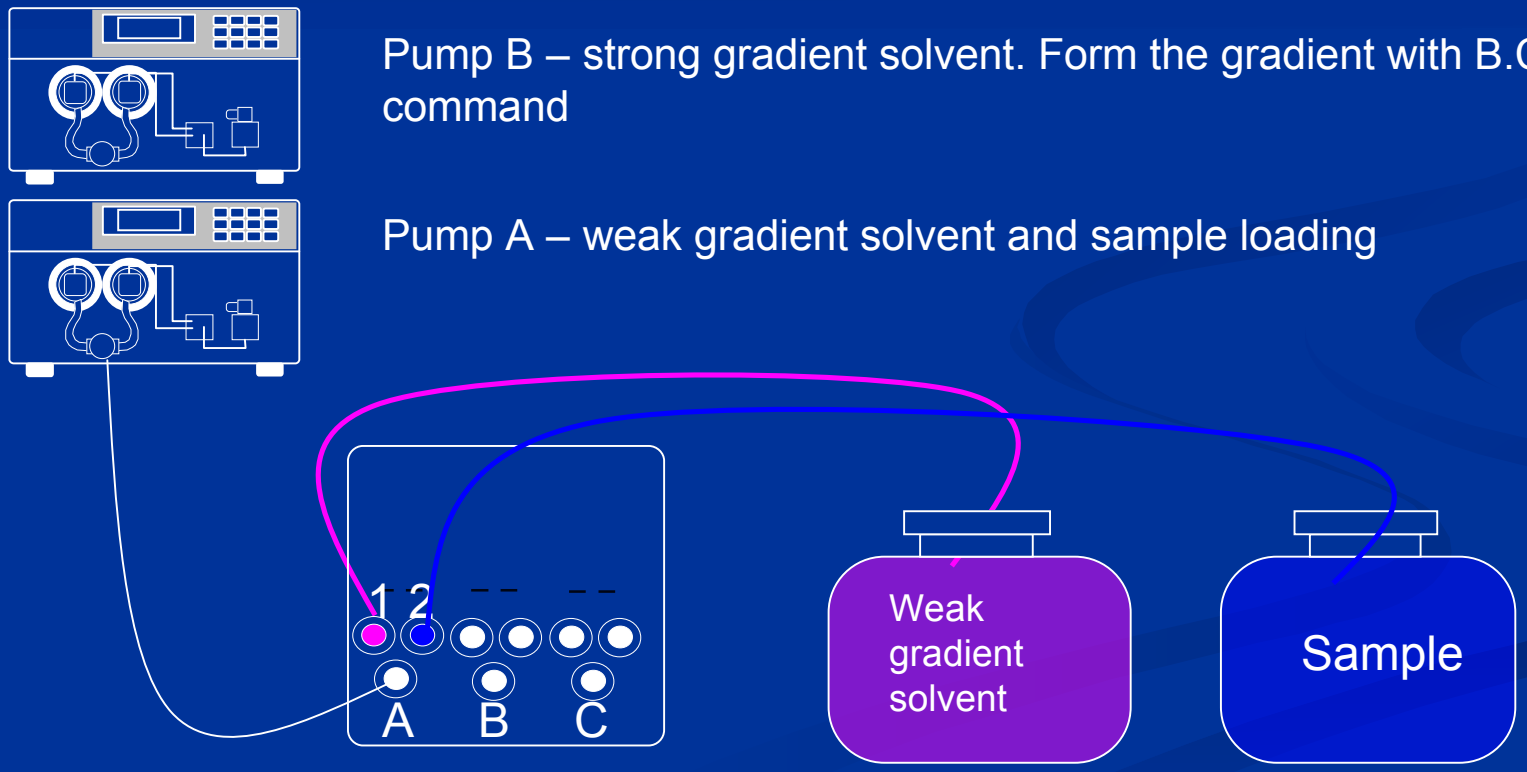
Valves Used With Pumps

- Solvent Selection – 2 Solvents Per Pump
  - Use for solvent switching



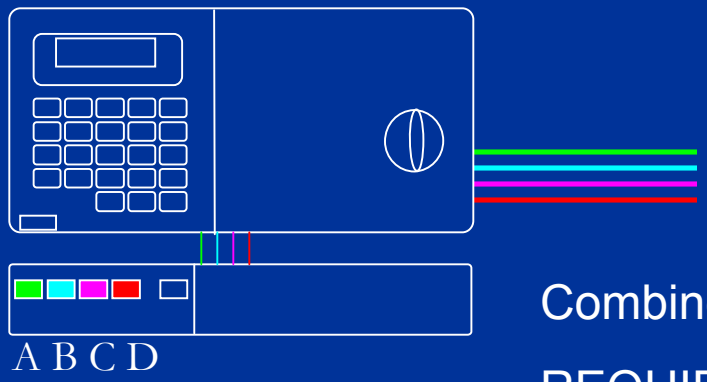
Valves Used With Pumps

- Solvent Selection – 2 Solvents Per Pump
  - Use for pump loading of large sample volumes



Valves Used With Pumps

- Solvent Selection – 4 Solvents Per Pump
  - Use for low pressure gradient formation

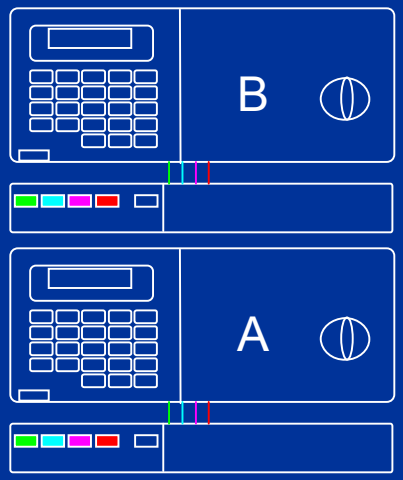


Combine any proportion of A/B/C/D.

REQUIRES additional mixing before the injector.

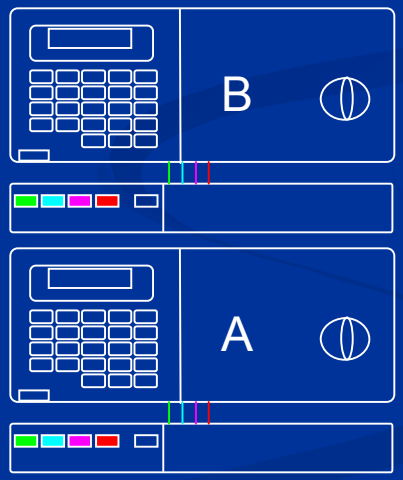
Valves Used With Pumps

- Solvent Selection – 4 Solvents Per Pump
  - Use for different gradients in method development



Pump A	Pump B
A	A
B	B
C	C
D	D

4 Pairs

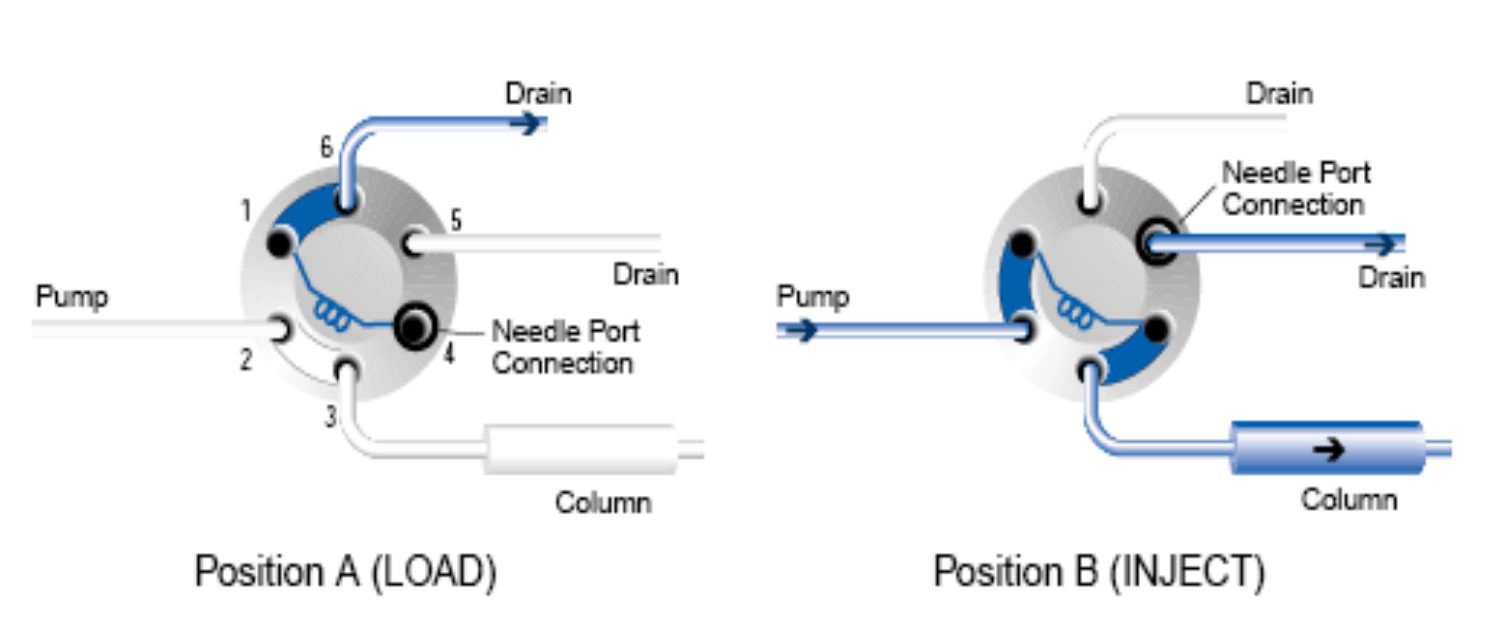


Pump A	Pump B
A	A, B, C, D
B	A, B, C, D
C	A, B, C, D
D	A, B, C, D

16 Combinations

Sample Injection – Manual

- Manual Injector with Syringe
  - Fixed loop of varying sizes (1 to 20 mL or more)
  - Fill with syringes of varying sizes
  - Can include a switch to start a data system

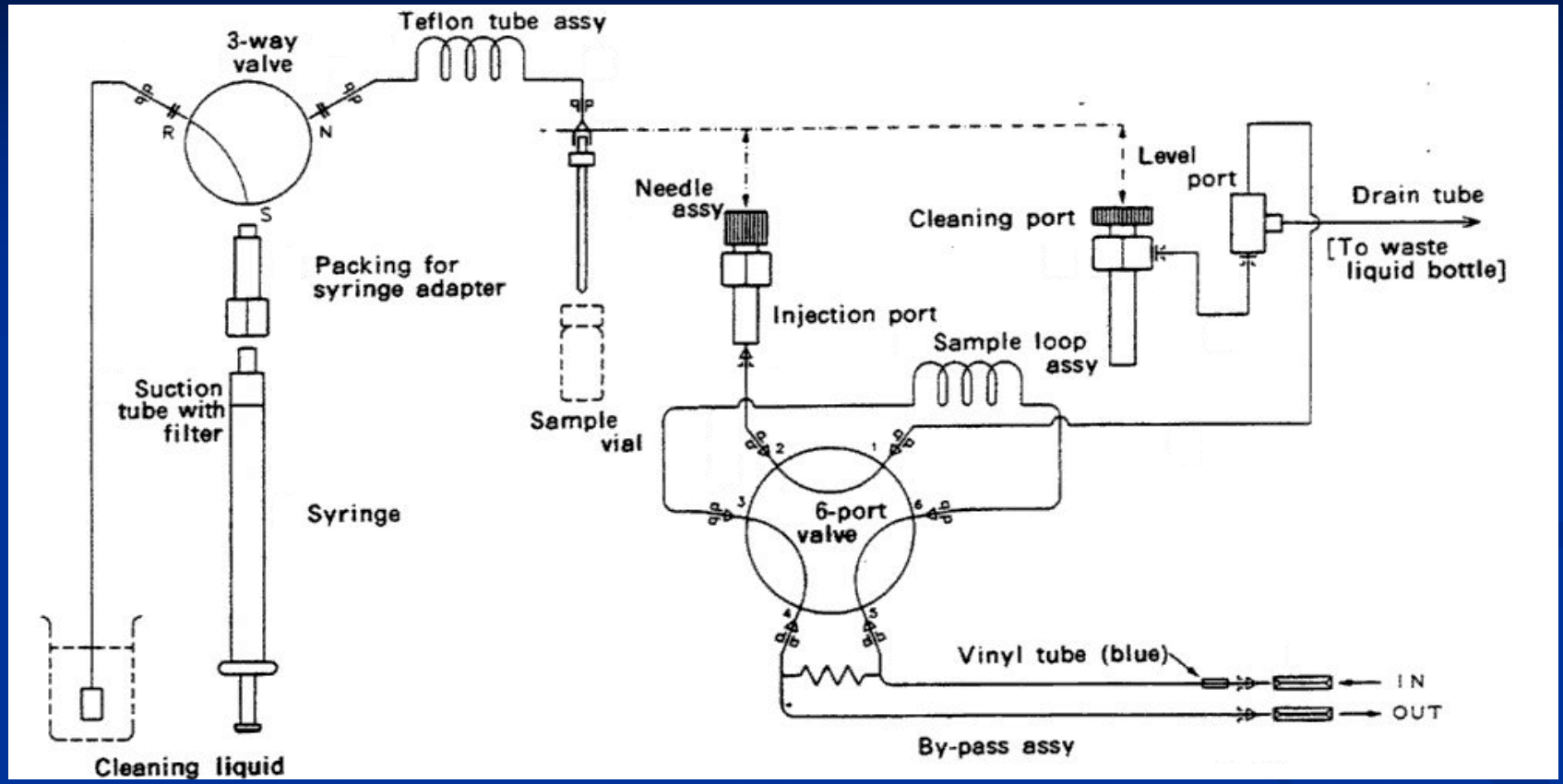


Picture from http://www.rheodyne.com/products/fluidic/manualapps/manualsample.asp#

Sample Injection – Automatic

- Fixed-Loop Autosampler
  - Loop is installed on the valve and can be changed for different injection volumes
  - External syringe draws sample and fills loop
- Advantages: low cost, rugged, few moving parts
- Disadvantages: Poor performance for low volume injections, higher carryover, always some sample loss

Sample Injection – Fixed Loop

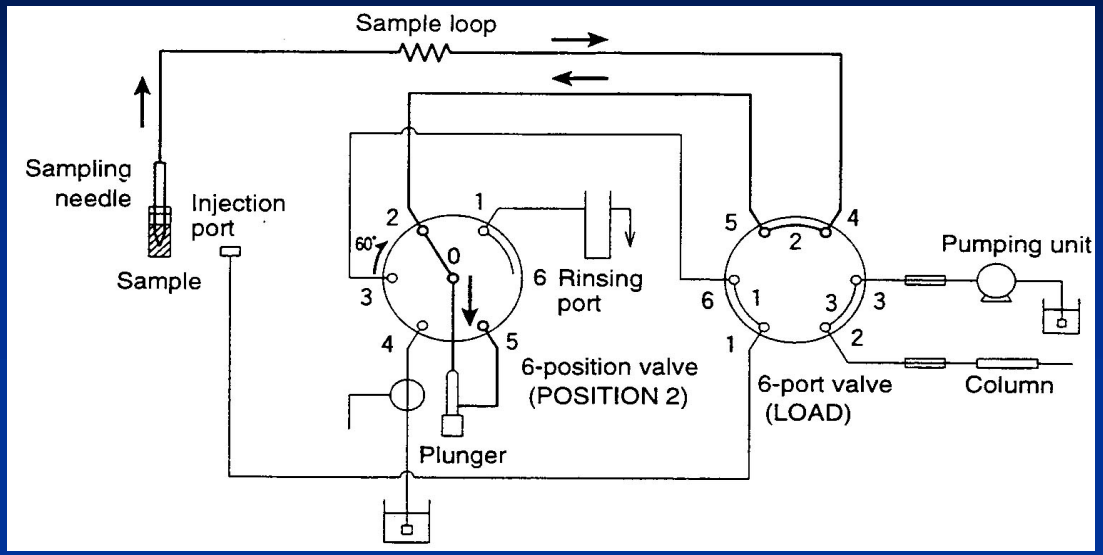


- External syringe draws sample, then fills the fixed-volume loop attached to the valve.

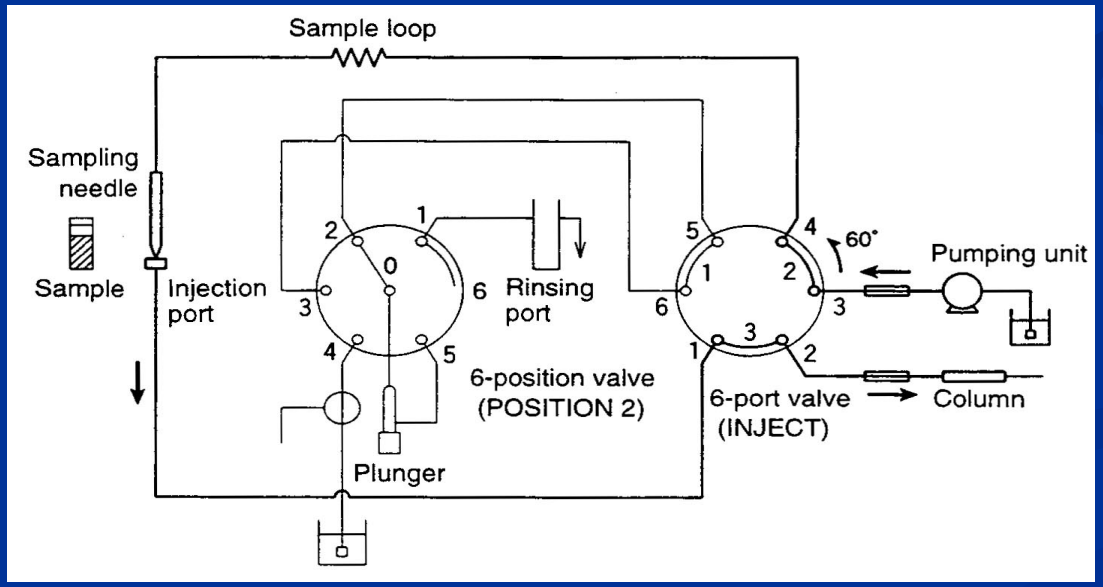
Sample Injection – Automatic

- Needle-in-the-flowpath autosampler
  - Sample loop and needle are a single piece of tubing
  - Loop and needle are cleaned during the run
  - Metering pump draws sample very precisely
- Advantages: no sample loss, low carryover
- Disadvantages: higher cost, more delay volume for gradient

Sample Injection to Flow Path

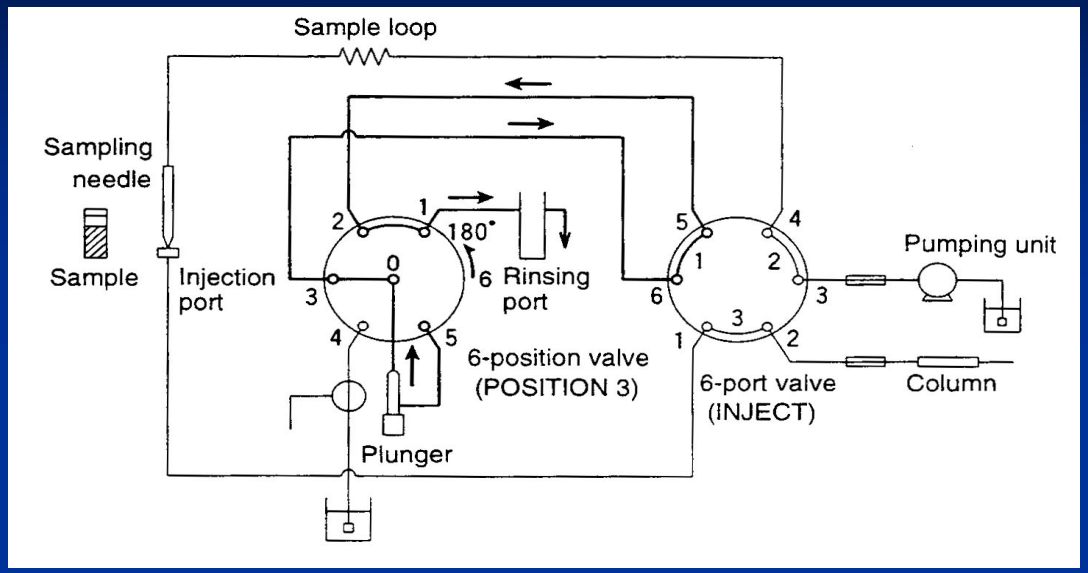


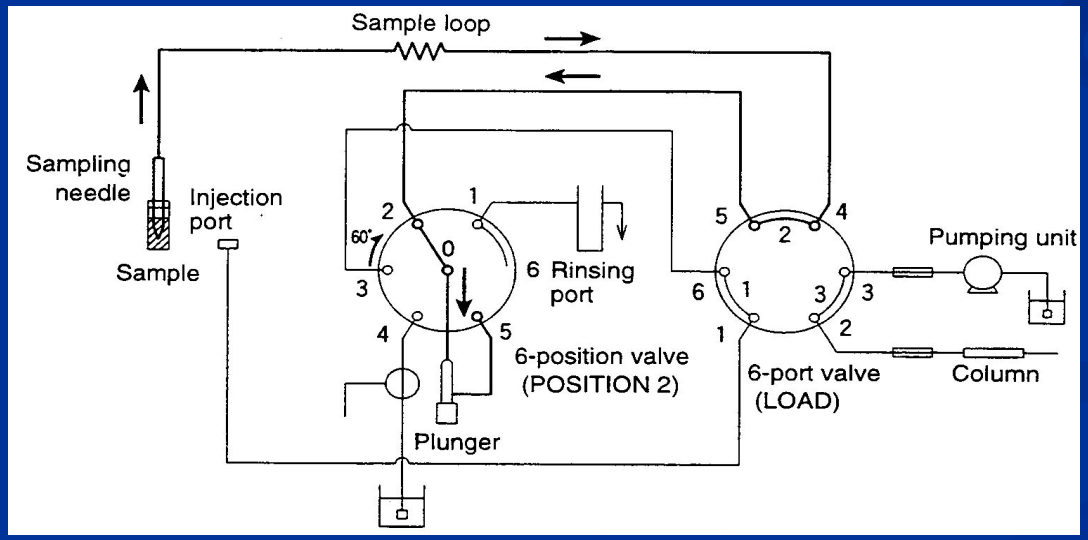
- Sample Loading



- Sample Injection – Everything drawn into the needle goes to the column.

Rinsing After Injection





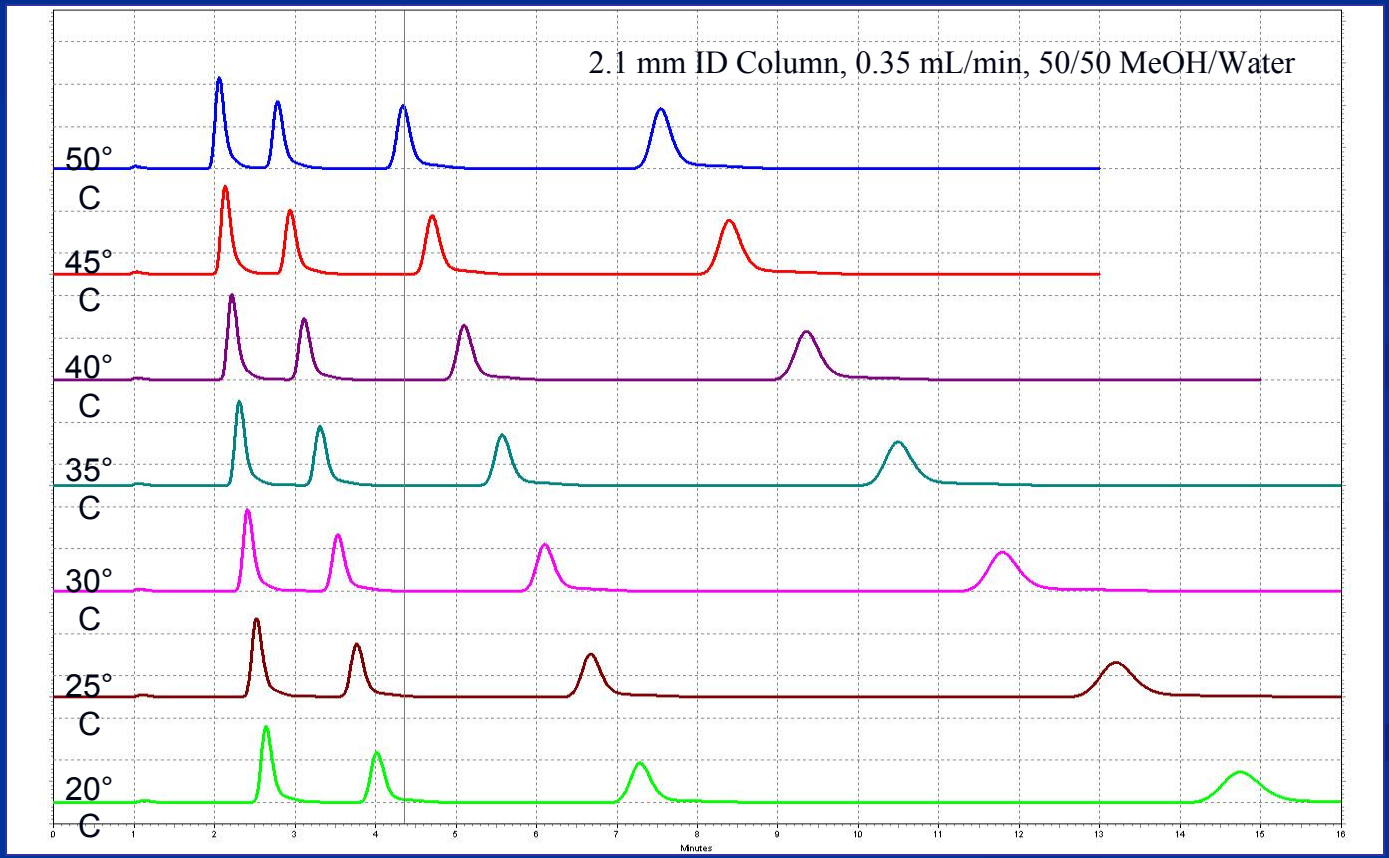
- Rinse liquid flows through ports 5 and 6 of the high pressure valve.
- Sample aspiration uses port 5.
- If air is present around port 5, injection reproducibility will be low.
- Rinse liquid MUST be degassed!

HPLC Column Ovens

- Block heater with solvent preheater
  - Column is housed between 2 metal plates
  - Mobile phase is plumbed into the block for preheating
- Forced air
  - Column is in a large chamber with air circulation
  - Better temperature equilibration
  - Room for column switching valves

Why Use a Column Oven?

- Retention times decrease, and higher flow rates are possible



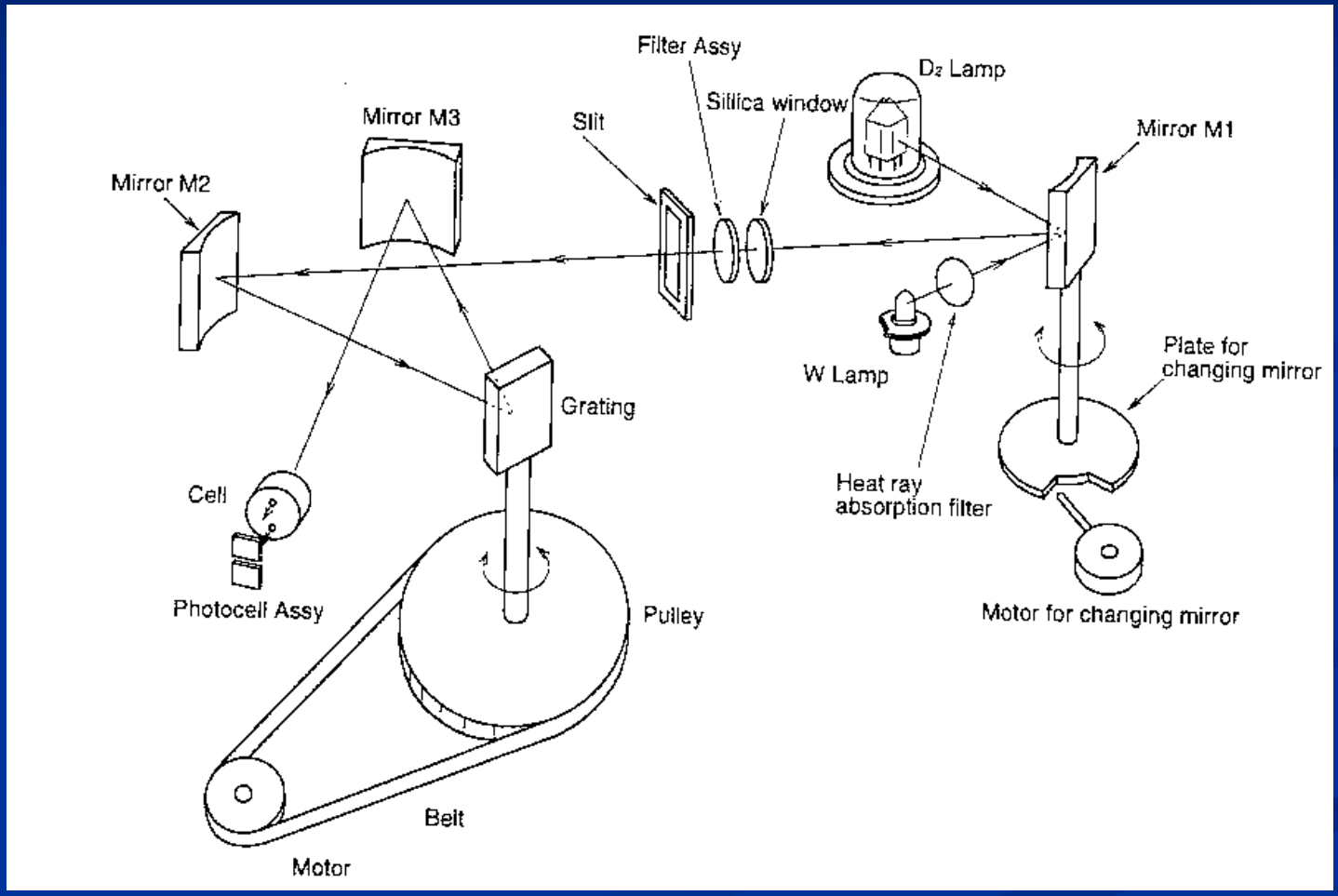
HPLC Detectors

- UV-VIS
- Diode Array
- Refractive Index
- Fluorescence
- Light Scattering
- Conductivity
- Mass Spectrometer

HPLC Detectors – UV-VIS

- UV-VIS
  - Wavelength range 190-700 nm
  - D2 and W lamps
- Most common HPLC detector for a variety of samples
  - Proteins and peptides
  - Organic molecules
  - Pharmaceuticals
- Monitor 2 wavelengths at one time

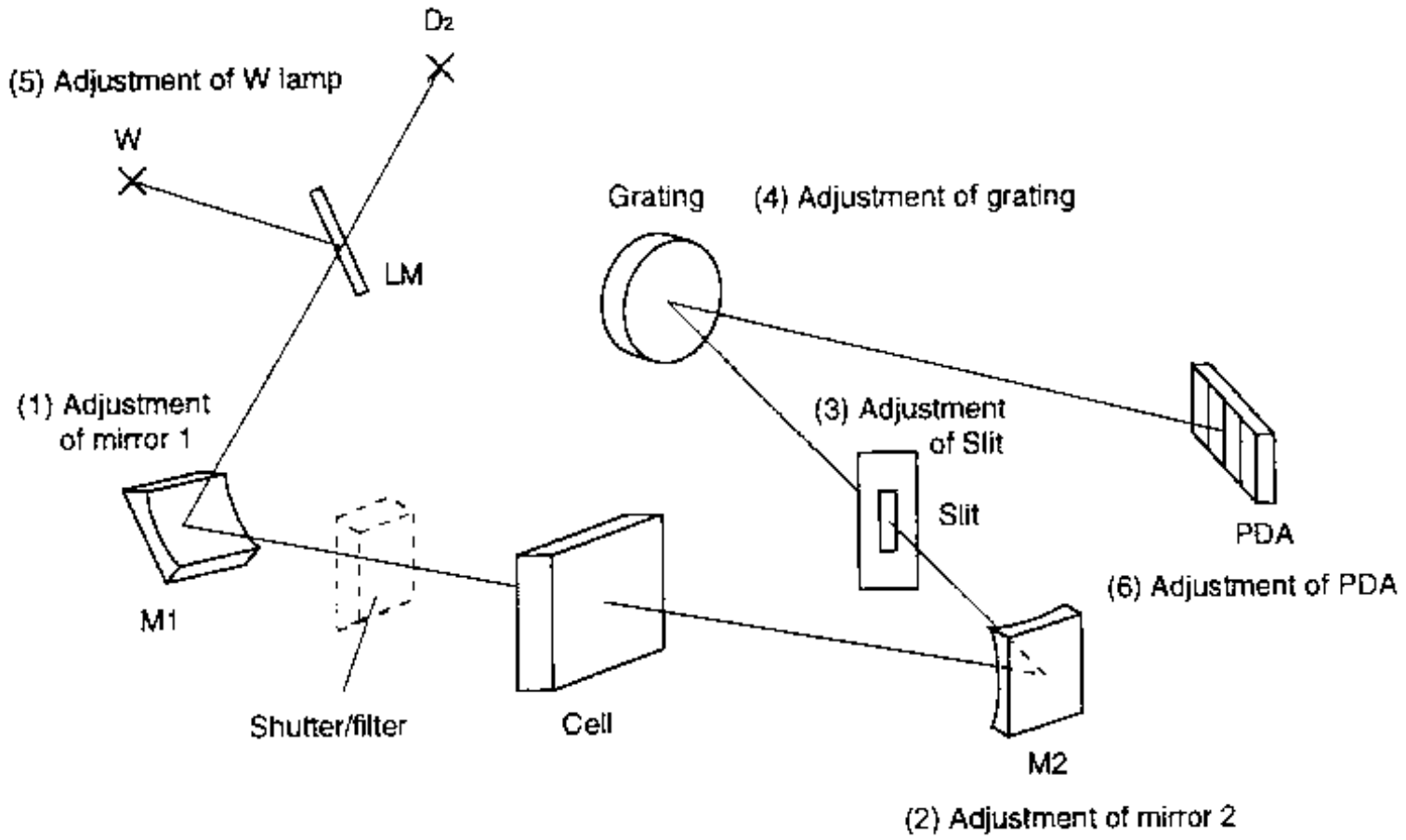
HPLC Detectors – UV-VIS



HPLC Detectors – Diode Array

- Diode Array
  - Wavelength range 190-900 nm
  - D2 and W lamps
- Spectral information about sample
- Create compound libraries to identify unknowns
- Monitor an entire wavelength range at one time – up to 790 wavelengths vs. only 2 with a UV detector

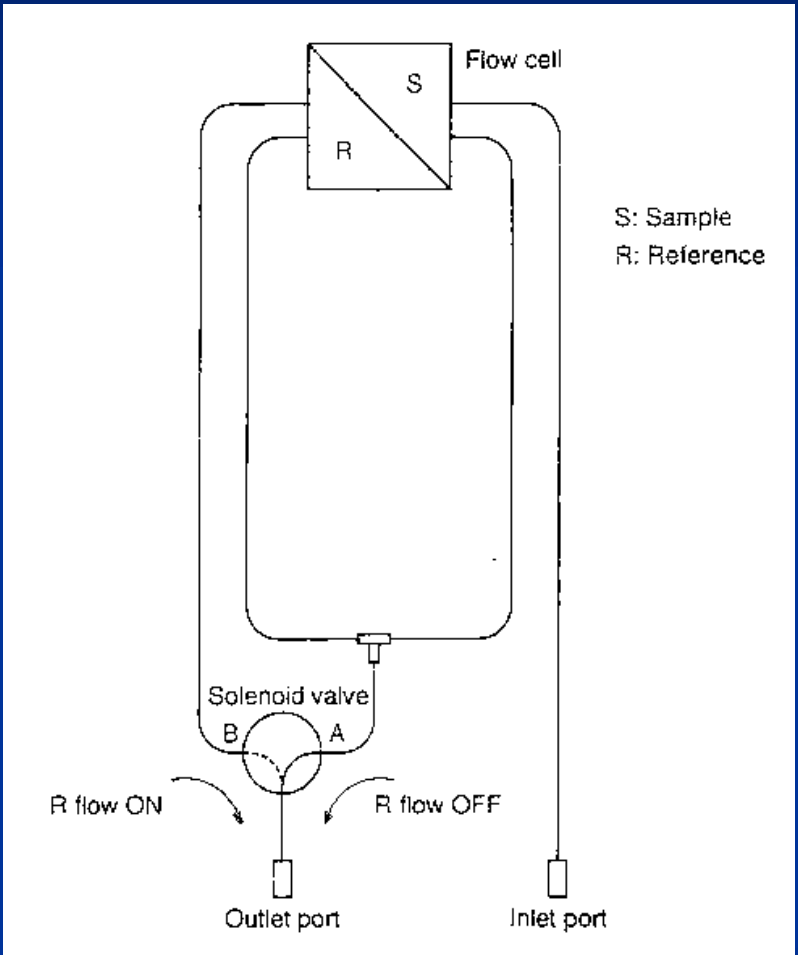
HPLC Detectors – Diode Array



HPLC Detectors

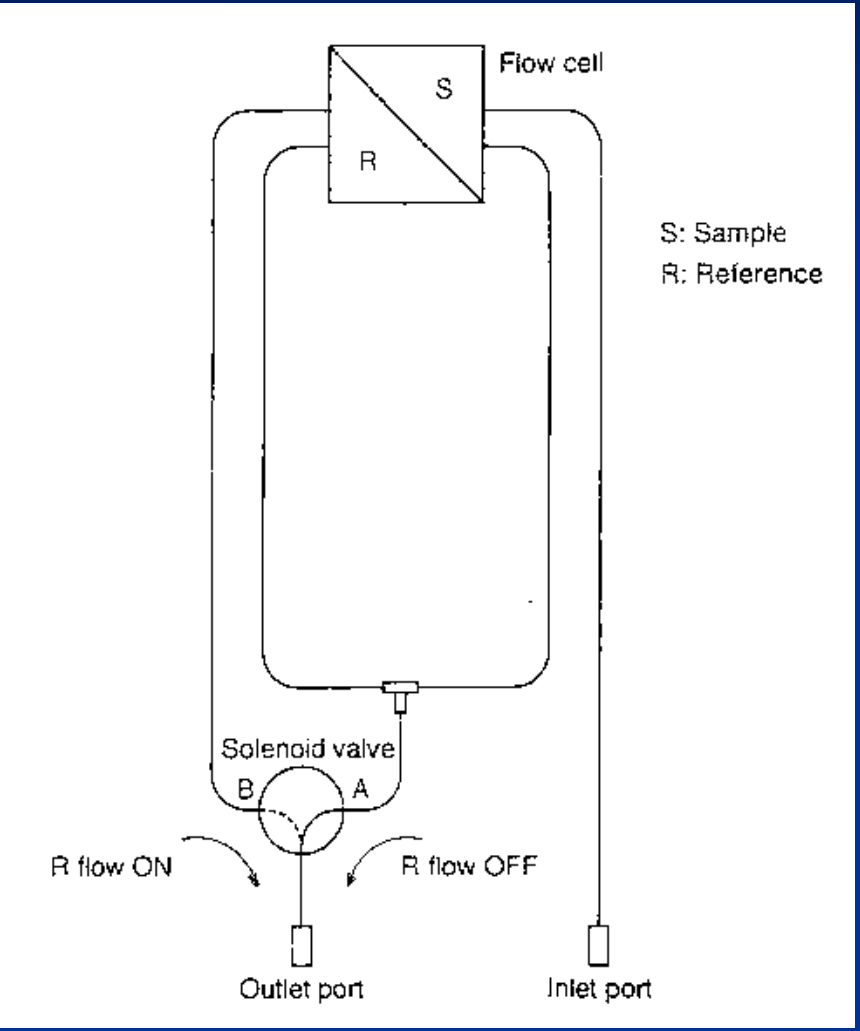
- Refractive Index
  - For samples with little or no UV Absorption
    - Alcohols, sugars, saccharides, fatty acids, polymers
  - Best results when RI of samples is very different from RI of mobile phase
  - Flow cell is temperature controlled with a double insulated heating block.
- REQUIRES isocratic separations
- REQUIRES low pulsation pumps

HPLC Detectors – RI Balance



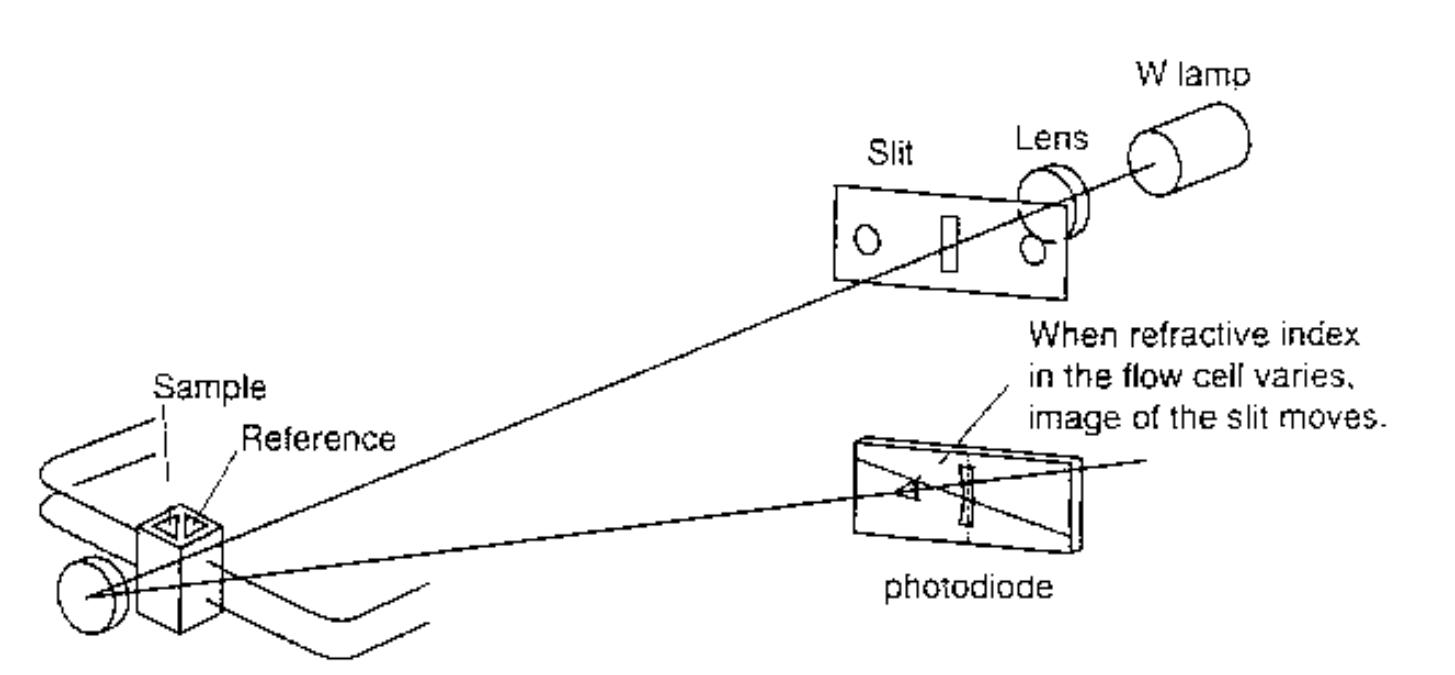
- Fill sample and reference cell with mobile phase.

HPLC Detectors – RI Analyze



- Mobile phase flows through sample side only.

HPLC Detectors – RI Analyze

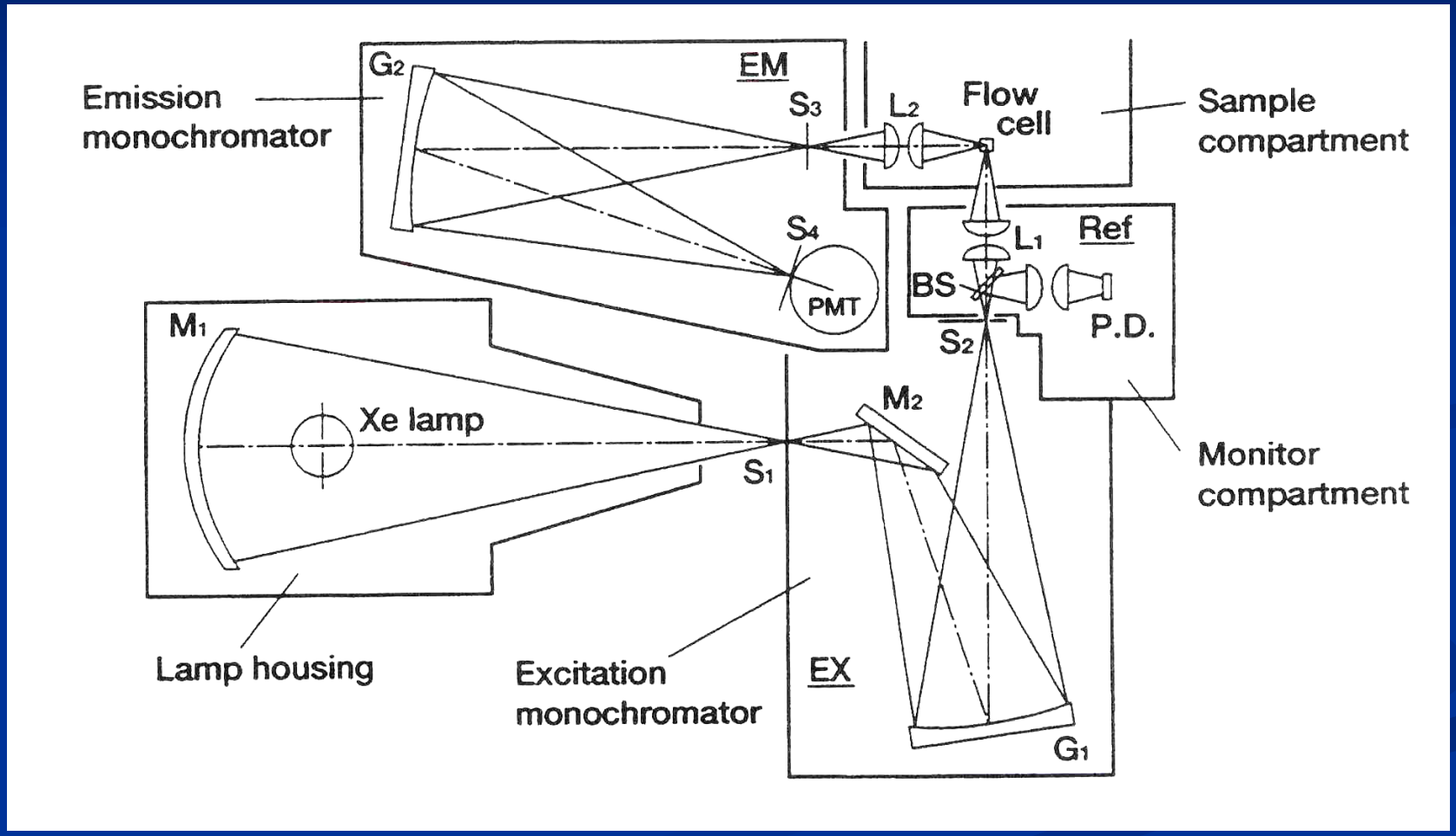


- As the refractive index changes, the image on the photodiode is deflected or "unbalanced", and the difference in current to the photodiode is measured.

HPLC Detectors

- Fluorescence
  - Xenon lamp for light source
  - Excitation wavelength range: 200-650 nm
  - Emission wavelength range: up to 900 nm depending on photomultiplier installed
- Used primarily for amino acid analysis
  - Derivatize samples before (pre-column) or after separation(post-column)

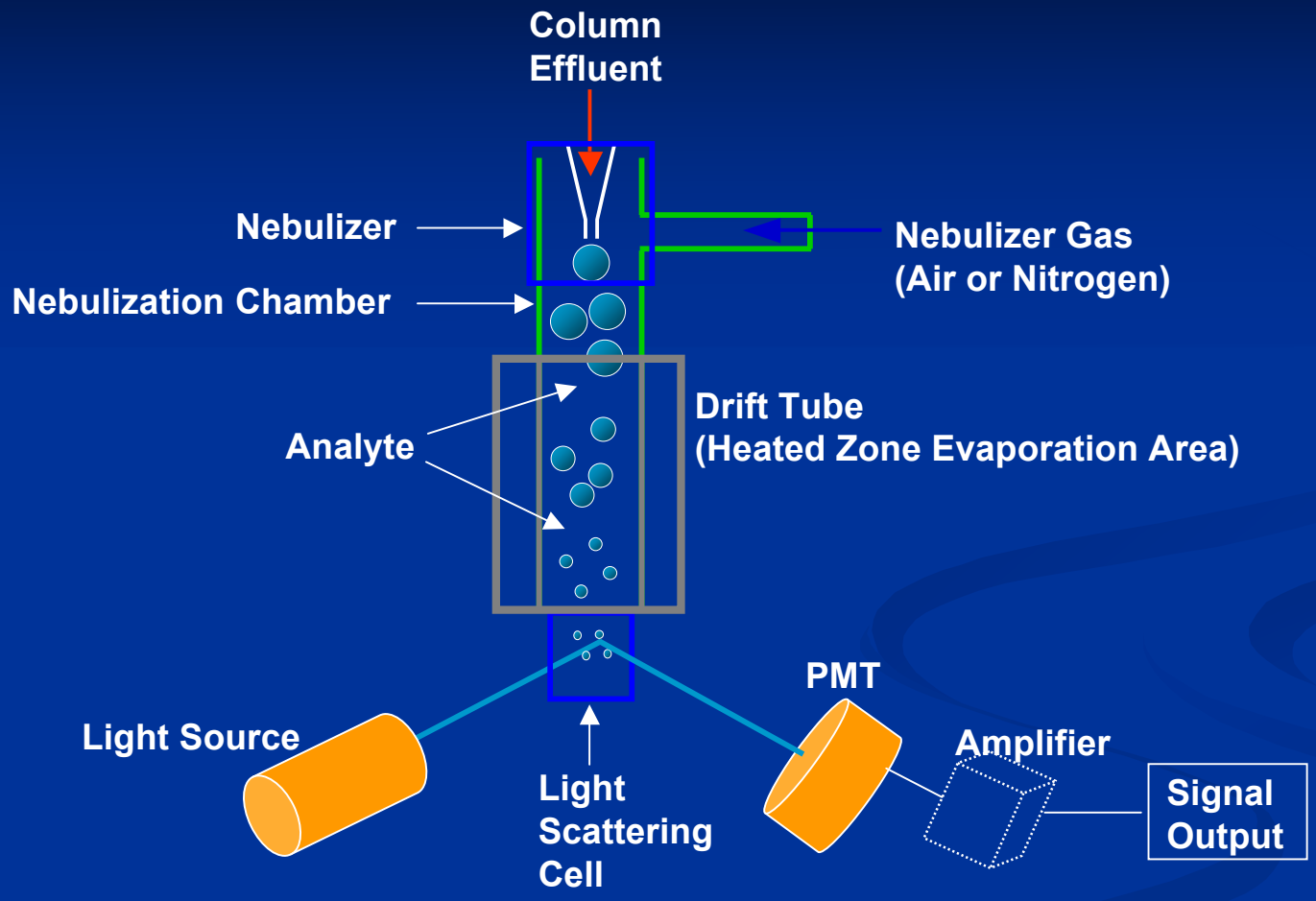
HPLC Detectors - Fluorescence



HPLC Detectors

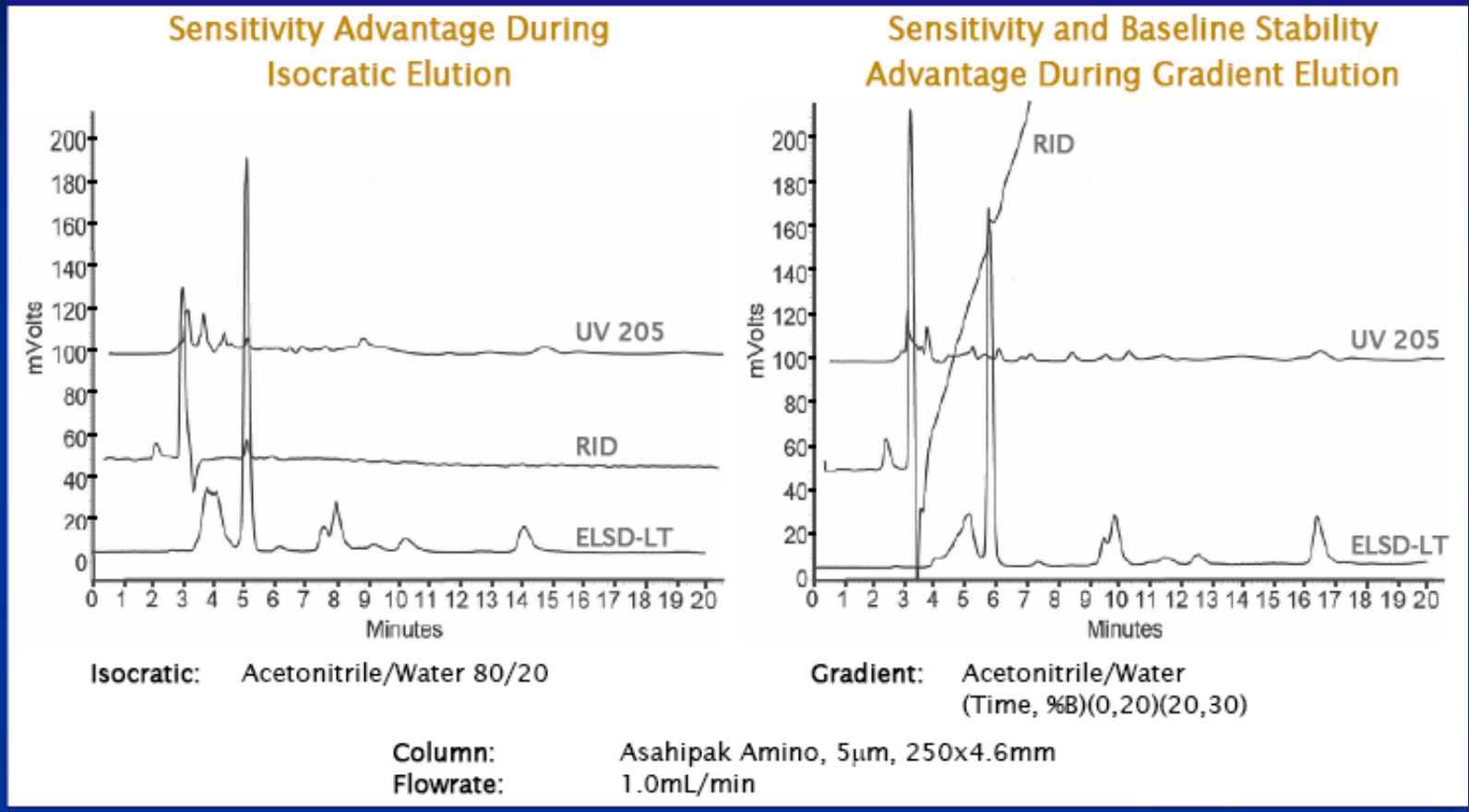
- Evaporative Light Scattering (ELSD)
  - Also for low or no UV absorbing compounds
    - Sometimes called a "Universal" detector
  - Requires NO equilibration (unlike RID)
  - Can be used with gradients and volatile buffers (unlike RID)
  - Semi-volatile compounds can be detected at low temperatures

ELSD Operation



ELSD vs. Other Detectors

Simultaneous ELSD-LT, RID and UV for Carbohydrates



- ELSD has higher sensitivity than UV and RID
- ELSD can be used with gradients, unlike RID

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HPLC Detectors

- Conductivity
  - Flow cell contains 2 electrodes
  - Measure ion amounts in sample
- REQUIRES low pulsation pumps
- Flow cell must be placed in a column oven

HPLC Detectors - Conductivity

- Conductivity
  - Use in Environmental and water testing
    - Fl^-, Cl^- NO_3^-, PO_4^{3-}, SO_4^{2-}
    - Li^+, Na^+, K^+, Mg^{2+}, Cu^{2+}, M-CN complexes
  - Determine organic acids in fruit juice
    - Oxalic, Maleic, Malic, Succinic, Citric
  - Analyze surfactants
    - Sulfonates, long/short chain ammonium

HPLC Detectors

- Mass Spectrometer
  - Separate sample components as ions according to their mass to charge (m/z) ratio
- Three stages to detection
  - Vaporization: liquid from HPLC column converted to an aerosol
  - Ionization: neutral molecules converted to charged species (either positive or negative)
  - Mass Analysis: filter ions by m/z ratio

HPLC Detectors – Mass Spec

- Two Ionization Types
- APCI: Atmospheric Pressure Chemical Ionization
  - For molecules up to 1000 Da
  - Singly charges ions
  - Best for analysis of non-polar molecules
- ESI: Electrospray Ionization
  - Can be used for large biopolymers
  - Forms multiply charged ions
  - Best for the analysis of polar molecules, especially pharmaceutical products and proteins

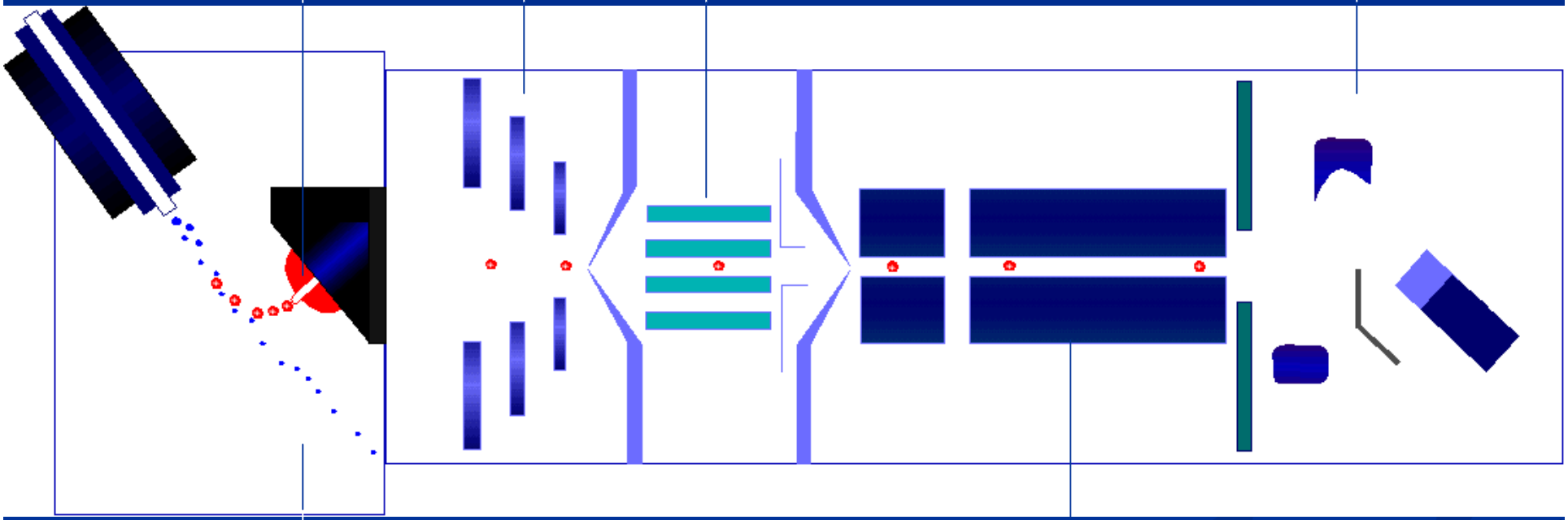
HPLC Detectors – Mass Spec

Heated capillary

Q-array

Octapole

Electron Multiplie Detector



Orthogonal source geometry

Quadrupole mass analyser

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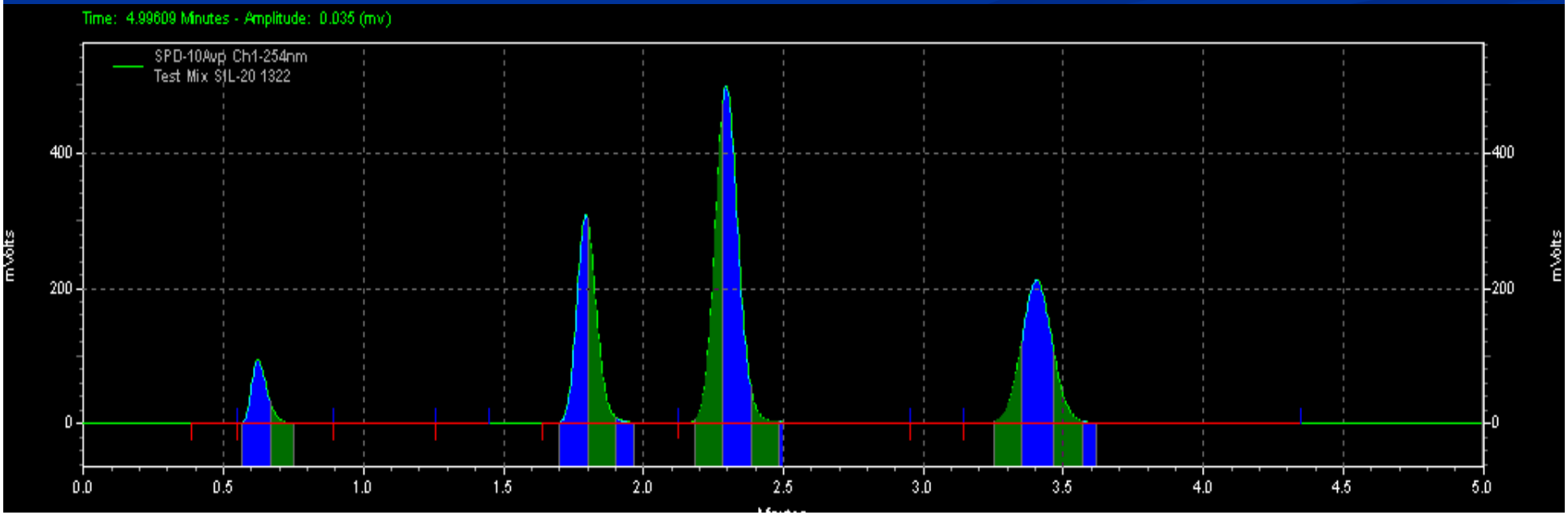
HPLC System Components

- Fraction Collector
  - Purify raw materials or compounds from synthesis
  - Collect by slope, level, time, volume
  - Isolate single peaks per tube, or divide peaks into small “slices” for extra purity



Questions About Hardware Components??

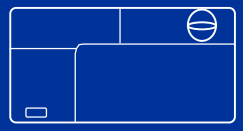
Next – HPLC System Types. Now that we have hardware components and columns, what do we DO with them??

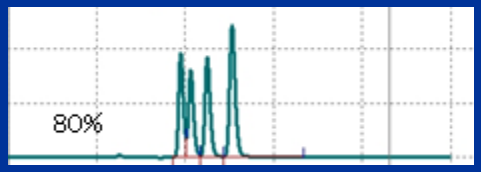
HPLC System Types

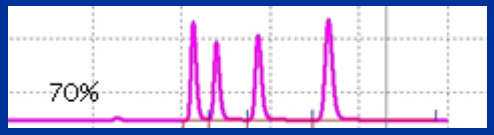
- Isocratic system
  - Same mobile phase concentration throughout the separation
  - Use 1 pump and pre-mix solvents
  - Use 1 pump and a valve for 4 different solvents
  - Use 2 pumps and vary the amount coming from each pump

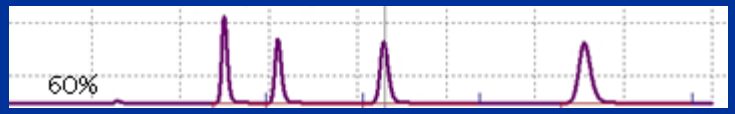
Isocratic Separation

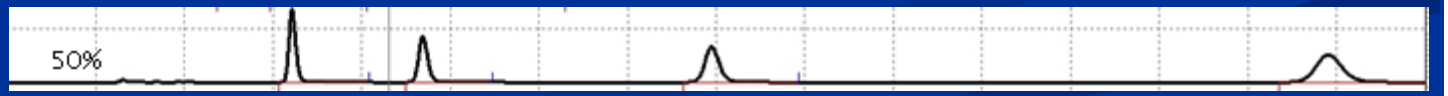
- 1 pump and premixing
- 4.6 mm ID Column, 1 mL/min, Changing MeOH % vs Water











Isocratic Separation

- 1 pump with valve and premixing

A = 80% Methanol, 20% Water

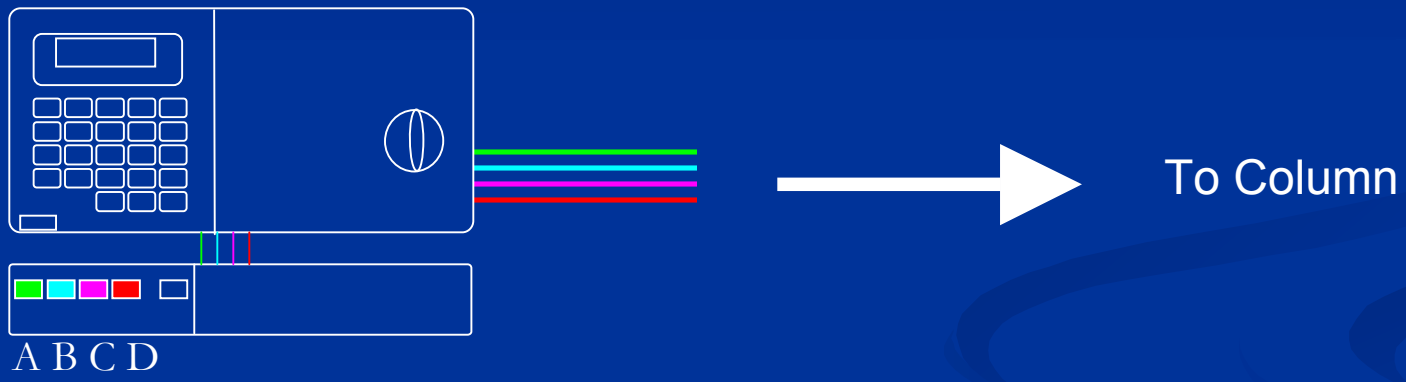
B = 70% Methanol, 30% Water

C = 60% Methanol, 40% Water

D = 50% Methanol, 50% Water

Isocratic Separation

- 1 pump with mixer – let the pump do the work!



Method 1: A.CONC = 20%, B.CONC = 80%

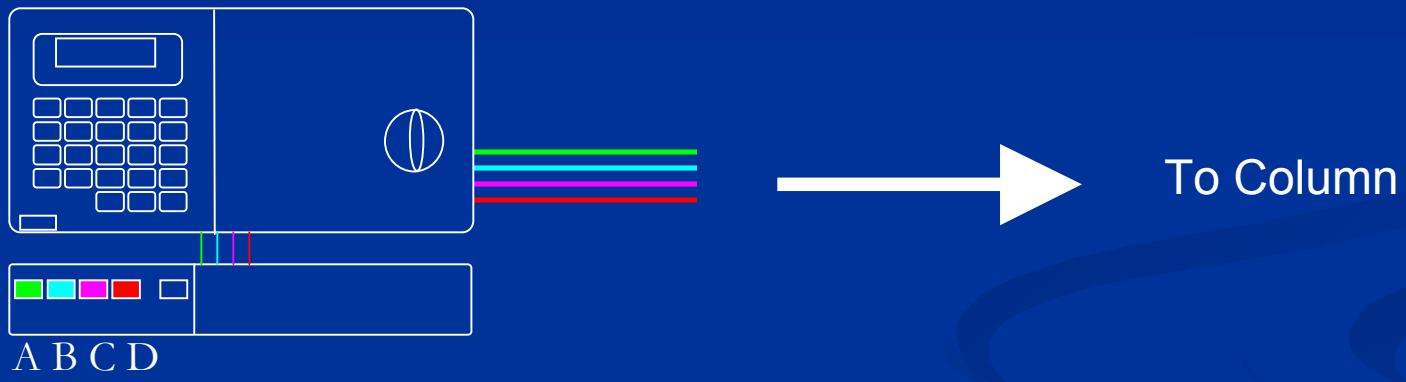
Method 2: A.CONC = 30%, B.CONC = 70%

Method 3: A.CONC = 40%, B.CONC = 60%

Method 4: A.CONC = 50%, B.CONC = 50%

Low Pressure Gradient

- 1 Pump, solvents are mixed before the pump



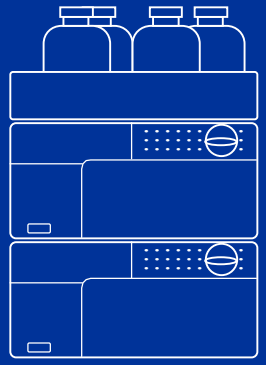
- REQUIRES degassing

HPLC System Types

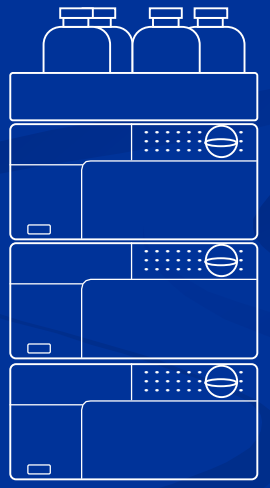
- High Pressure Gradient
  - Multiple pumps are used with a mixer after the pumps
- Low Pressure Gradient
  - Solvents are mixed before the pump

High Pressure Gradient

- Binary Gradient
- 2 Pumps and Mixer

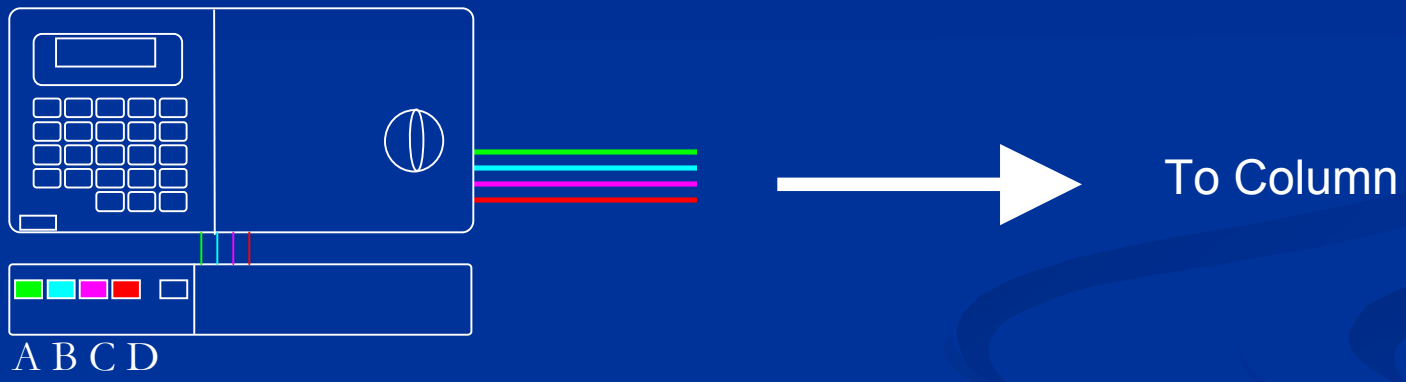


- Ternary Gradient
- 3 Pumps and Mixer



Low Pressure Gradient

- 1 Pump, solvents are mixed before the pump



- REQUIRES degassing

Questions About System Types?

Next: Troubleshooting and How to Take Care of Your Column and HPLC System

HPLC Troubleshooting

- Pressure: too much or too little
- Leaks: pump, autosampler, detector
- Reproducibility: pump, autosampler

- Column Care: Flushing and equilibration

Pump Troubleshooting

- No pressure, or fluctuating pressure
  - Pump may not be completely full of liquid – check solvent inlet line
  - Air in check valve – always degas mobile phase!
  - "Stuck" check valve – the pump may have been idle for too long and solvent has dried inside the check valve.

    Poor quality solvent: may contain resins that coat the ball inside the check valve, and that film won't let the ball seat properly

Pump Troubleshooting

- High Pressure
  - Outlet frit may be blocked with particles from mobile phase or seal material
- Leaks
  - Damage to seal and/or plunger due to several factors
    - Misaligned plunger
    - Solvent incompatibility with seal material
    - Salt crystal buildup from buffers – use a rinse kit!

Pump Troubleshooting

- Retention Time Reproducibility
  - For a dual piston pump, only one side may be filled with liquid – check solvent inlet lines
  - Temperature change (may not be the pump's fault)
    - A 1° shift in temperature can result in a 1-2% shift in retention time
    - Avoid drafty locations in the lab
    - Use a column oven when possible

Autosampler Troubleshooting

- High Pressure
  - Particulates from mobile phase, sample, pump may be trapped in the inlet tubing or valve
    - Filter mobile phase AND sample when possible
- Leaks
  - Fittings may be loose on the valve
    - Tighten fittings properly and don't exceed the pressure limit of the autosampler

Autosampler Troubleshooting

- Area % Reproducibility
  - Always degas rinse phase, and use some volume of liquid for rinsing to keep all flow paths in the valves full of liquid
  - Make sure the needle stroke is deep enough to draw sample from the vial
  - Check for leaks on the valve fittings, and the connection to the column inlet

Detector Troubleshooting

- Spiky Baseline
  - Air bubble in flow cell – degas mobile phase!
    - Put some restriction on the cell outlet, but not too much! Tubing with 0.005" i.d. is fine.
- Leaks
  - Cracked flow cell
    - Don't exceed the pressure limit of the cell
  - Poor tubing connections
    - Use the proper fittings and tighten appropriately

Column Care

- Follow MFR's recommendations for solvent compatibility, flow rate, and pressure limits
- Filter samples when possible
  - Particulates will build up on the inlet frit over time
- Use care when reversing column flow
  - Connect the outlet to waste, NOT inline with the detector to prevent further contamination
- Store columns in recommended solvents

Troubleshooting Summary

- Throw away bad parts and columns.
- Leaks do not fix themselves.
- If it doesn't pass, you must degas.

Questions About Troubleshooting?

Tomorrow: Application-Specific Systems, Software, and Prominence Demonstration

HPLC Applicated Systems

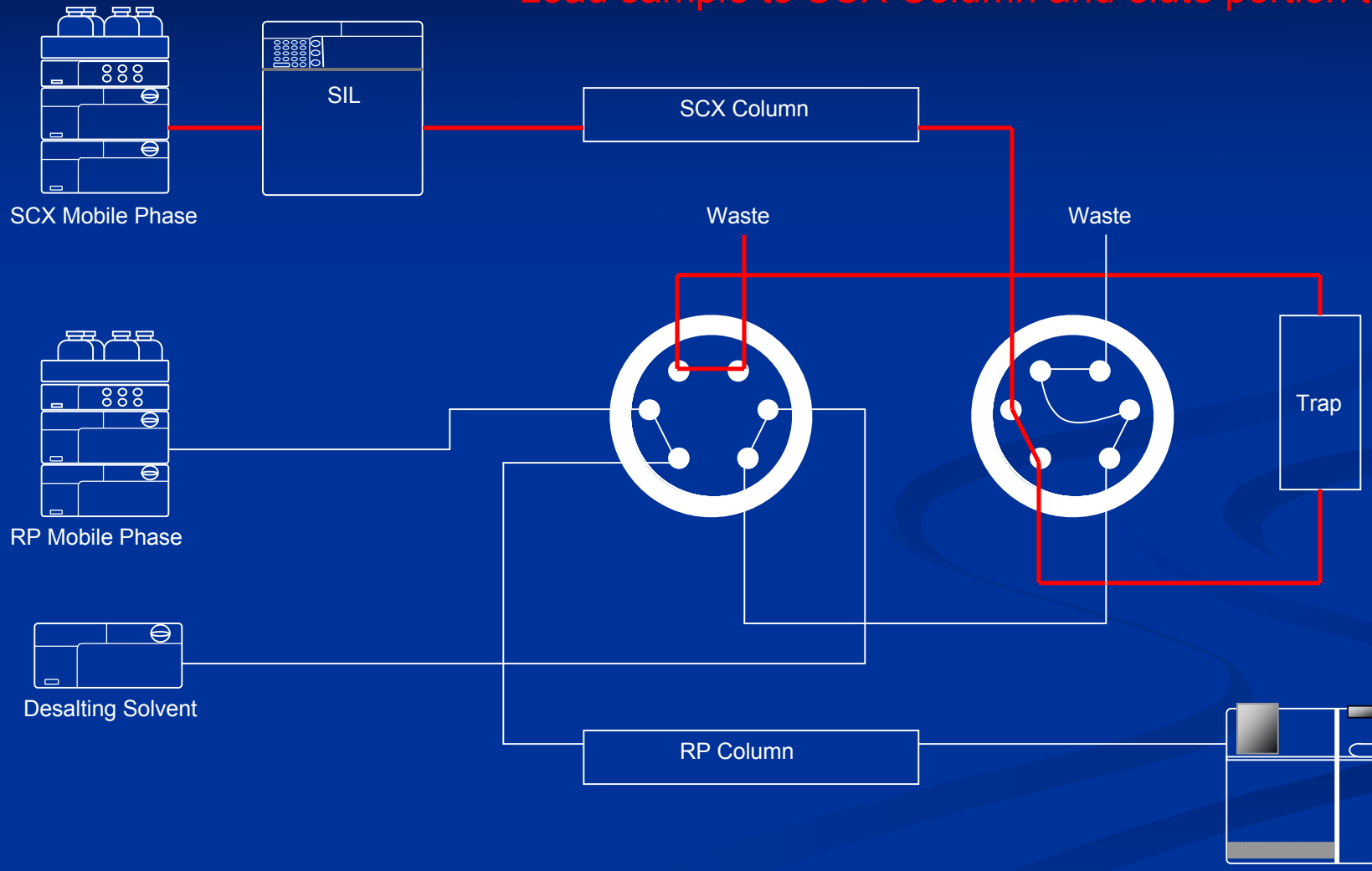
- Protein Separations
  - Column selection is important: reversed phase C-18, ion exchange most common
  - Buffered mobile phases often used so a rinse kit for the pumps is recommended
  - Inert (PEEK) pump and autosampler may be necessary
  - UV or Diode Array detection
  - Fraction collection for isolation and purification

HPLC Applicated Systems

- Proteomics
  - Very small sample amounts with many components
  - Use 2-dimensional chromatography
    - Elute portions of sample onto a trap column with a salt gradient
    - Desalt the trap then transfer sample to reversed phase column
    - Elute with a reversed phase gradient

2-Dimensional HPLC

Load sample to SCX Column and elute portion to Trap



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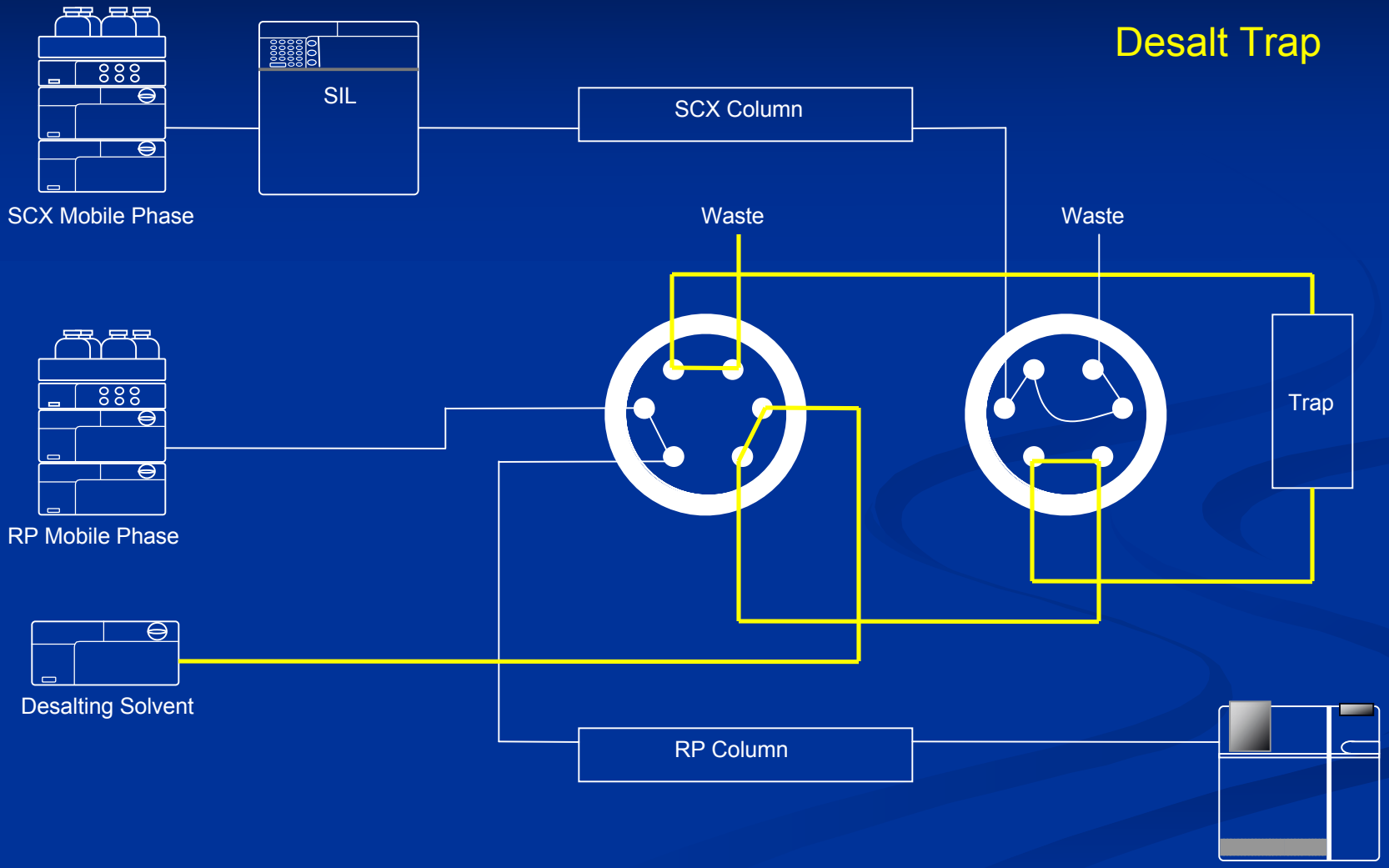
2-Dimensional HPLC



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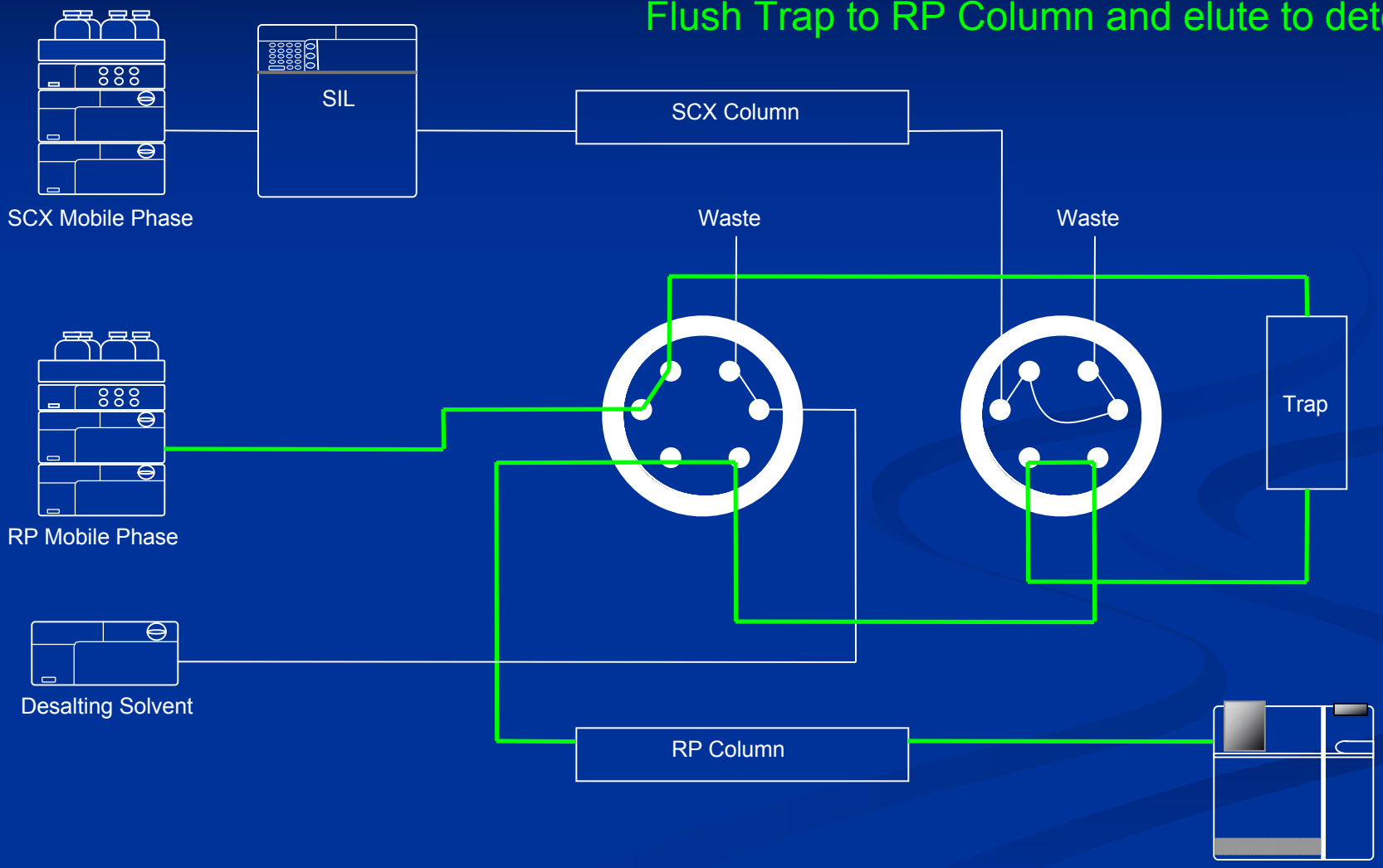
2-Dimensional HPLC



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HPLC Applicated Systems

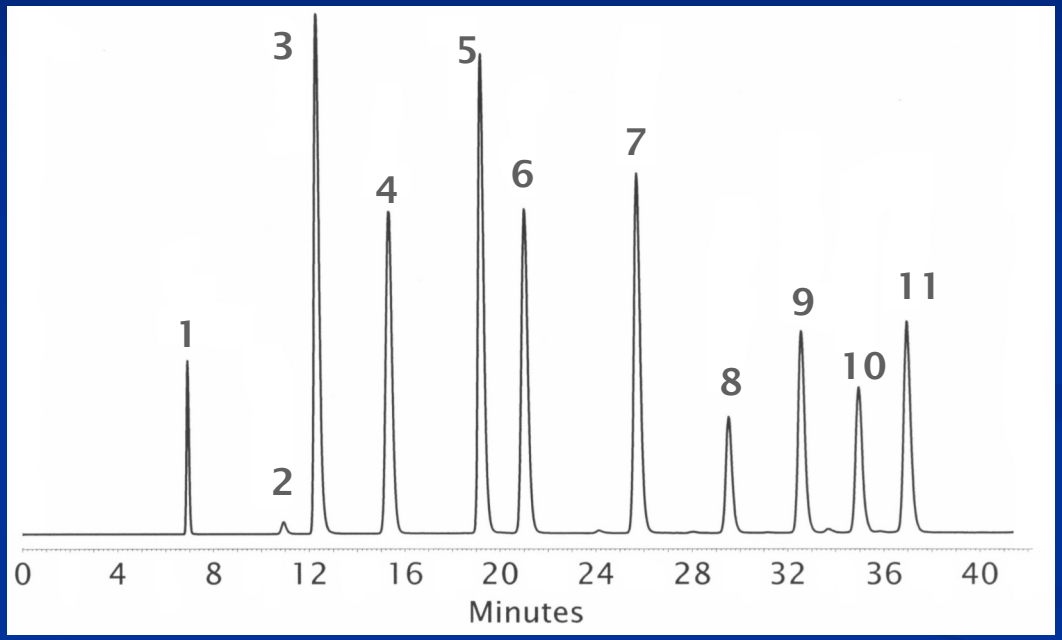
- Amino Acid Analysis
  - Column selection is important: C-18 is very common
  - Any pumps, autosampler, oven
  - Pre- or post column derivatization (OPA)
    - Autosampler can do pre-column reactions
    - Additional pump for post-column reagent addition
  - Fluorescence detection most common

HPLC Applicated Systems

- Food and Beverage Industry
  - Many isocratic methods
  - C18 columns, ion exchange columns
  - Any pumps, autosampler, oven
  - Traditional methods use UV, RID
  - Perfect opportunity for ELSD: App. notes on
    - Chili peppers
    - Wine
    - Sugar alcohols
    - Cereal

ELSD for Food and Beverage

Mono-, Di- and Oligosaccharide Standards

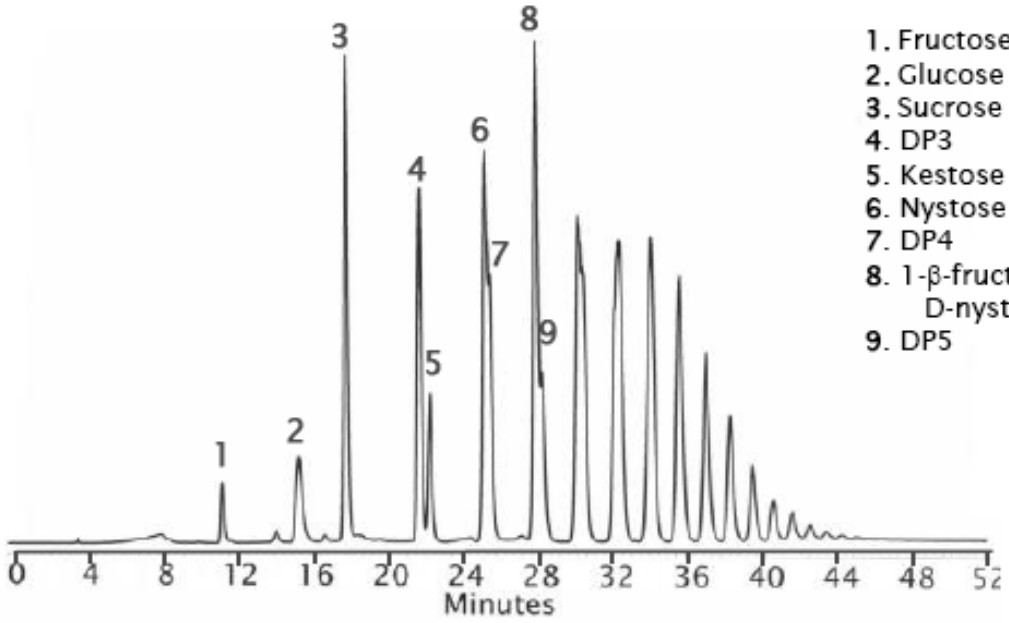


1. Glycerol
2. Arabinose
3. Fructose
4. Glucose
5. Sucrose
6. Maltose
7. Maltotriose
8. Maltotetraose
9. Maltopentaose
10. Maltohexaose
11. Maltoheptaose

Column:	Asahipak NH_2-P50, 5μm, 250x4.6mm
Mobile Phase:	**A**:Acetonitrile **B**: 0.0004N NH_4OH
Gradient:	(Time, %B)(0,15)(60,65)
Flowrate:	1.0mL/min
Col. Temp:	30°C
Detector:	Shimadzu ELSD-LT (Gain 5; T 40°C; P 250kPa)

ELSD for Food and Beverage

Fructo-oligosaccharides in Reconstituted, Minced Onions

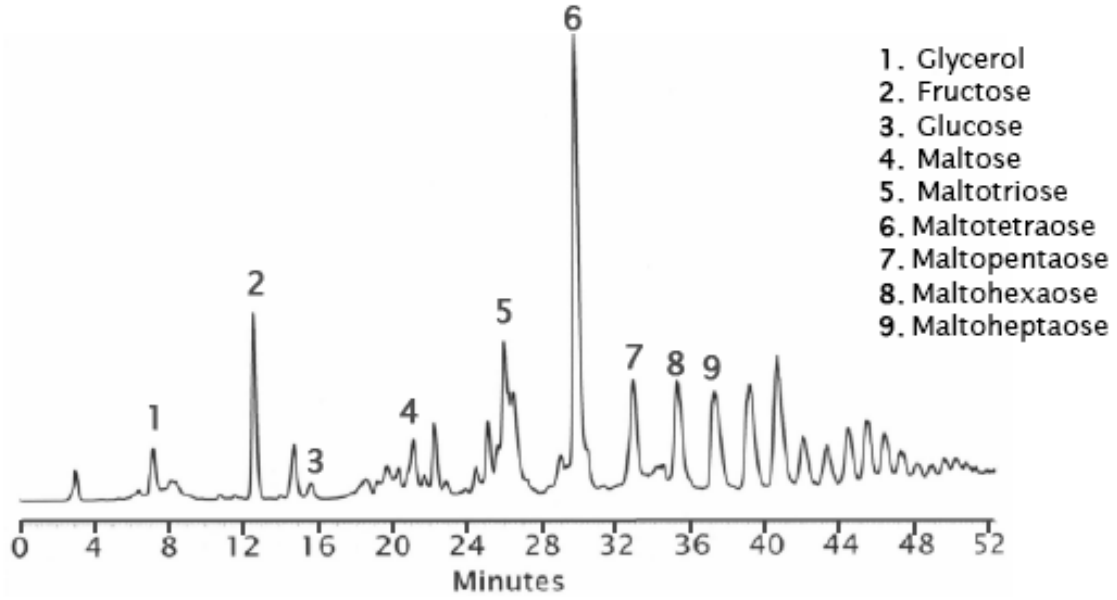


1. Fructose
2. Glucose
3. Sucrose
4. DP3
5. Kestose (DP3)
6. Nystose (DP4)
7. DP4
8. 1-β-fructofuranosyl-D-nystose (DP5)
9. DP5

Column:	Asahipak NH_2-P50, 5μm, 250x4.6mm
Mobile Phase:	**A**:Acetonitrile **B**: 0.0004N NH_4OH
Gradient:	(Time, %B)(0,15)(60,65)
Flowrate:	1.0mL/min
Col. Temp:	30°C
Detector:	Shimadzu ELSD-LT (Gain 6; T 40°C; P 250kPa)

ELSD for Food and Beverage

Malto-oligosaccharide Profile of Domestic Ale



Column:	Asahipak NH_2-P50, 5μm, 250x4.6mm
Mobile Phase:	**A**:Acetonitrile **B**: 0.0004N NH_4OH
Gradient:	(Time, %B)(0,15)(60,65)
Flowrate:	1.0mL/min
Col. Temp:	30°C
Detector:	Shimadzu ELSD-LT (Gain 8; T 40°C; P 250kPa)

HPLC Applicated Systems

- Nutraceutical: $46.7 BILLION In 2002, predicted to grow almost 10% each year*.
- Watch for these keywords
  - Functional foods/beverages
  - Fortified
  - Energy/nutrition
  - Health-promoting
  - Natural/Herbal
  - Vitamin/Mineral/Supplement

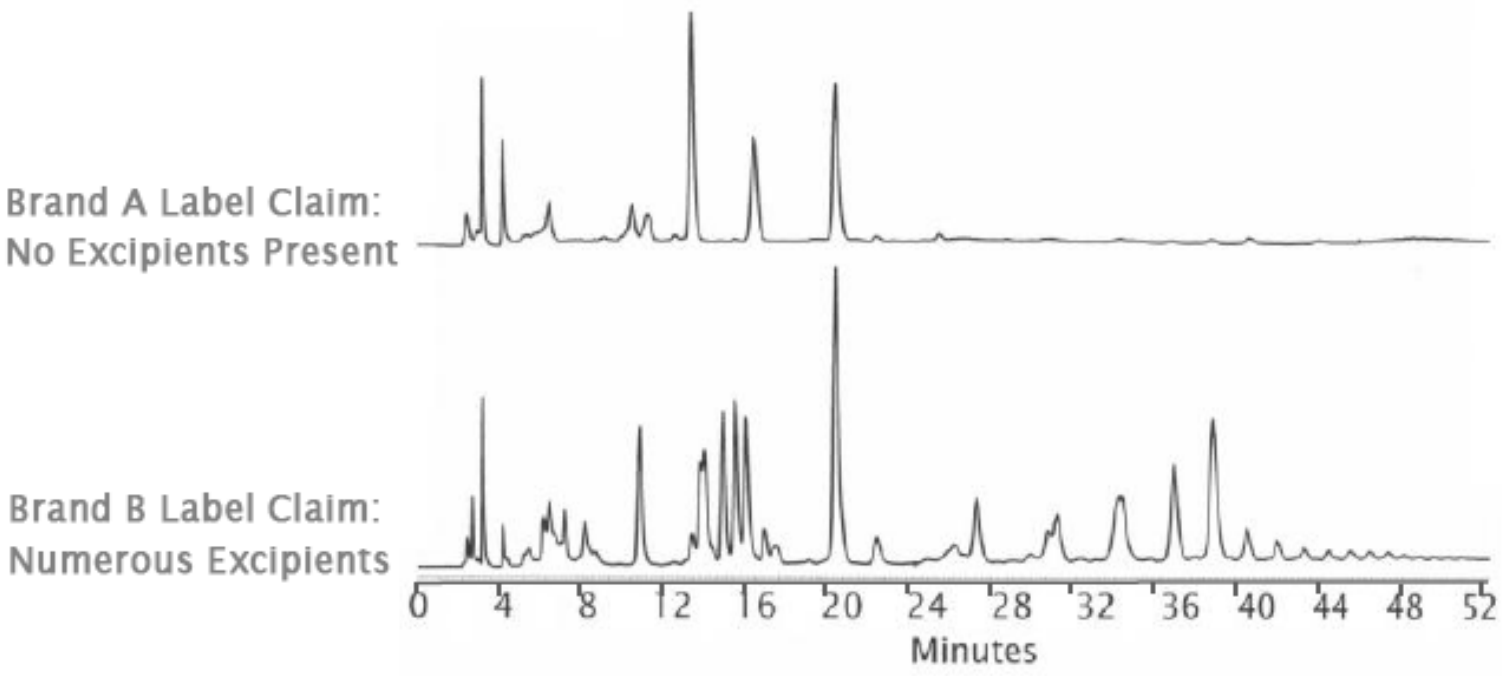
* http://www.bccresearch.com/editors/RGA-085R.html

HPLC Applicated Systems

- Nutraceutical system configurations
  - Similar to Food and Beverage
  - Promote ELSD since many compounds have low (or no!) UV absorbance
- There are many application notes available for nutraceutical samples
  - White Willow Bark
  - Black Cohosh
  - Milk Thistle

ELSD for Nutraceutical

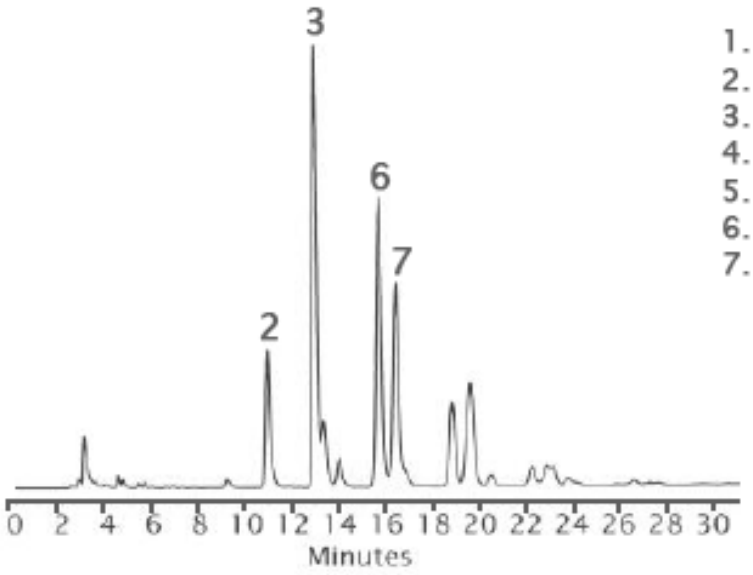
Siberian Ginseng Extract



Column:	Asahipak NH_2-P50, 5μm, 250x4.6mm
Mobile Phase:	A:Acetonitrile B: 0.0004N NH_4OH
Gradient:	(Time, %B)(0,15)(60,65)
Flowrate:	1.0mL/min
Col. Temp:	30°C
Detector:	Shimadzu ELSD-LT (Gain 5; T 40°C; P 250kPa)

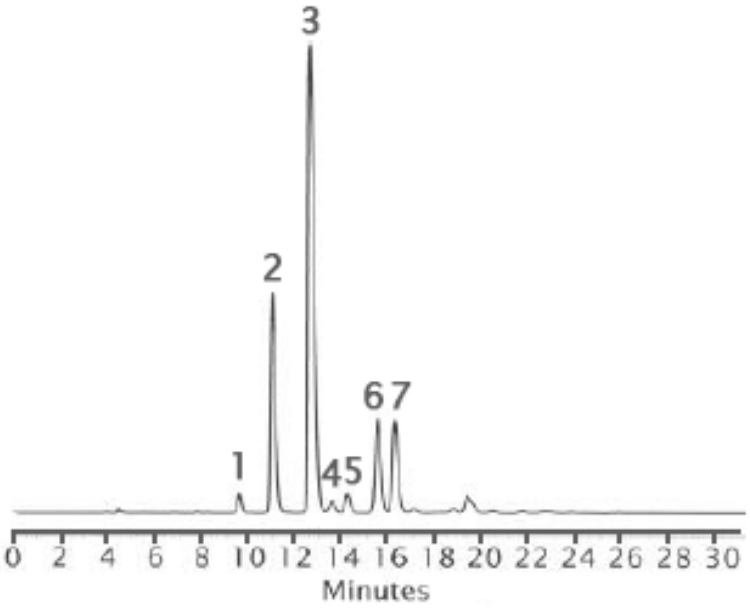
ELSD for Nutraceutical

Saw Palmetto Oil



1. LGG
2. LGL
3. LLL
4. OGL
5. PGL
6. OLL
7. OGO

Evening Primrose Oil



Column:	Shimadzu Premier C18, 5µm, 150x4.6mm
Mobile Phase:	A: Acetonitrile B: Dichloromethane
Gradient:	(Time, %B)(0,15)(20,30)(40,70)
Flowrate:	1.0mL/min
Col. Temp:	30°C
Detector:	Shimadzu ELSD-LT

HPLC Applicated Systems

- Ion Chromatography
  - Column selection is most important
  - Low pulsation pumps and any autosampler
  - UV or Conductivity detector
    - Ion chromatography applications data book
    - Suppressed or non-suppressed detection
      - Metrohm-Peak Model 833
      - Alltech Model 640 or 641

Ion Chromatography Applications

- Inorganic Anions – tap water
  - Fl-, Cl-, NO3-, PO43-, SO42-
- Cations and Transition Metals – tap water
  - Li+, Na+, K+, Mg2+, Cu2+, M-CN complexes
- Organic Acids – fruit juice
  - Oxalic, Maleic, Malic, Succinic, Citric
- Surfactants – soaps and detergents
  - Sulfonates, long/short chain ammonium

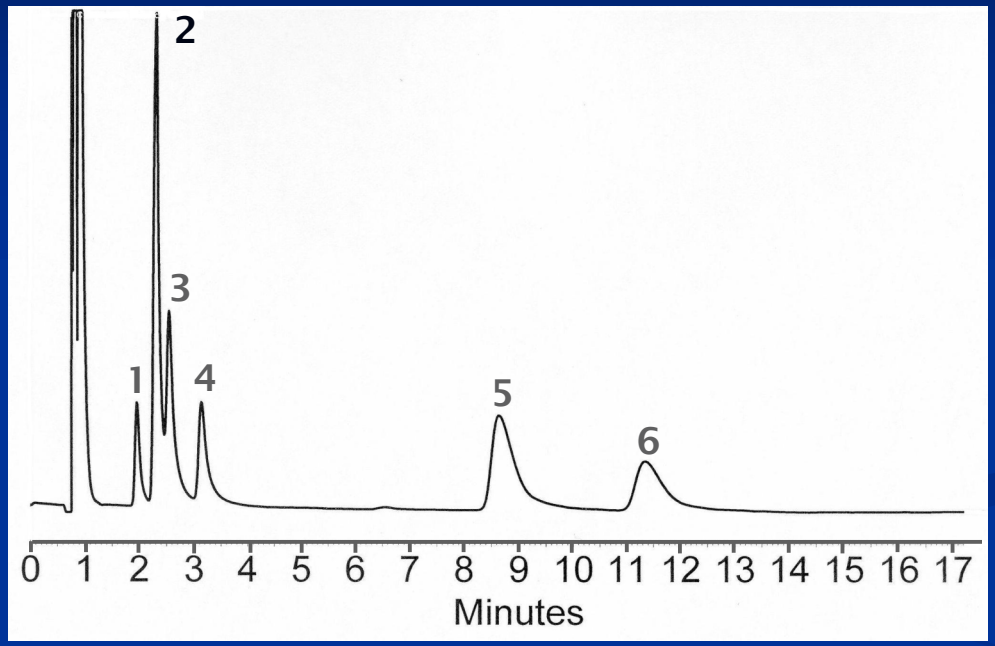
Ion Chromatography Columns

- Alltech
- Phenomenex
- Dionex

- Silica and polystyrene-based with specific functional groups

Ion Chromatography Applications

Common Cations

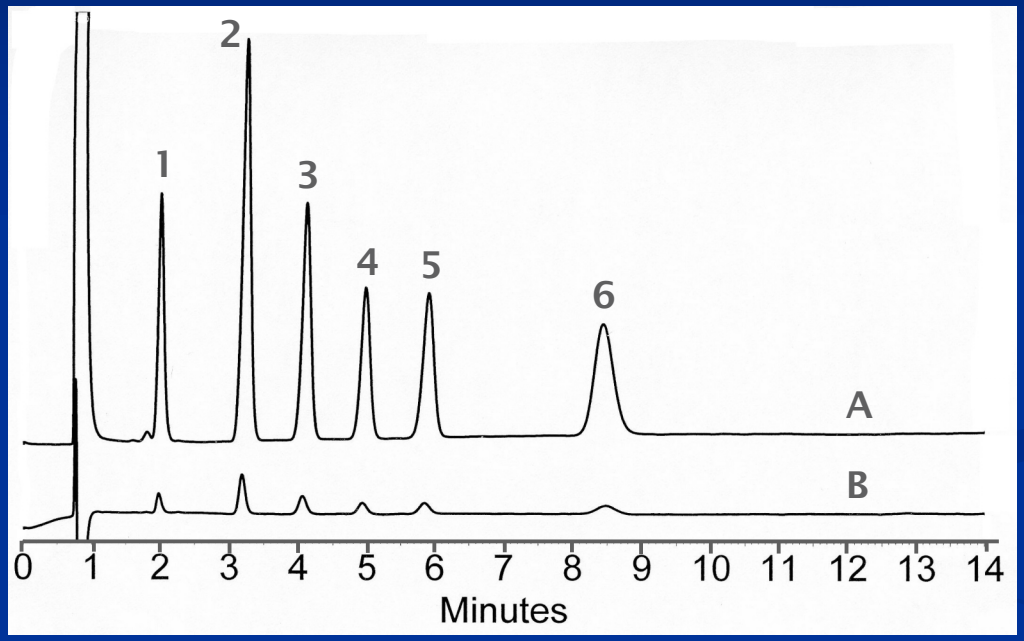


		(ppm)
1.	Potassium	2.5
2.	Magnesium	2
3.	Calcium	2
4.	Ammonium	1.5
5.	Sodium	1.5
6.	Lithium	0.2

Column:	ShimPak IC-C3, 5μm, 150x4.6mm
Mobile Phase:	2.5mM oxalic acid
Flowrate:	1.5mL/min
Col. Temp.:	40 ºC
Cell Temp.:	43 ºC
Inj. Vol.:	30μL
Detector:	Shimadzu CDD-10AVP non-suppressed (Gain 2; Polarity -1; Response 4)

Ion Chromatography Applications

Common Anions

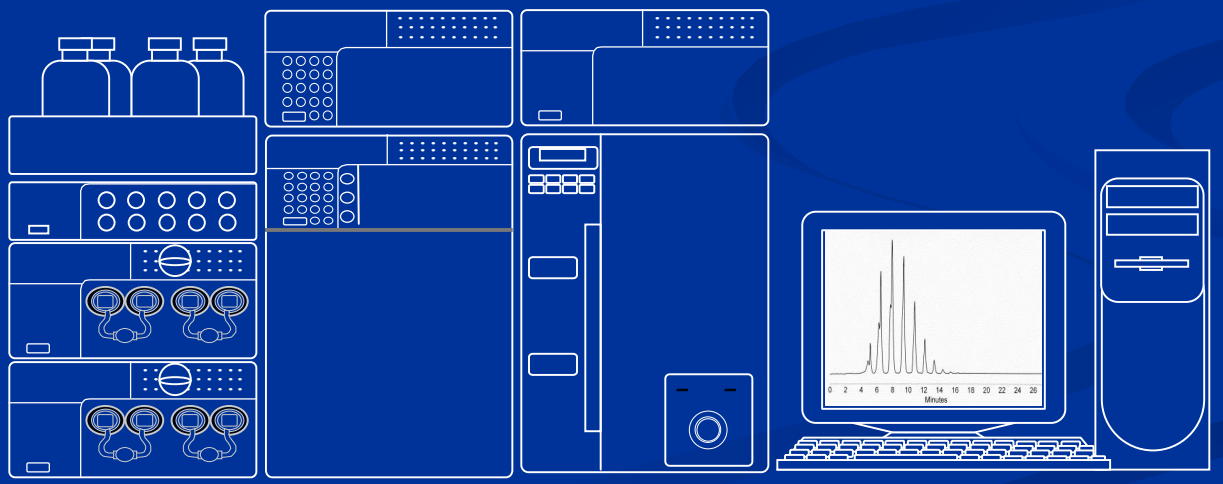


		A(ppm)	B(ppm)
1.	Fluoride	25	0.6
2.	Chloride	50	1.3
3.	Nitrite	50	1.3
4.	Bromide	50	1.3
5.	Nitrate	50	1.3
6.	Sulfate	50	1.3

Column: ShimPak IC-A3, 5μm, 150x4.6mm
Mobile Phase: 2mM phthalic acid @pH 4.2 with LiOH
Flowrate: 1.5mL/min
Col. Temp.: 37 ºC
Cell Temp.: 40 ºC
Inj. Vol.: 10μL
Detector: Shimadzu CDD-10AVP non-suppressed
(Gain 2; Polarity 1; Response 4)

Research and Method Development

- Typically, more “advanced” systems use multiple detectors and valves for column and solvent switching



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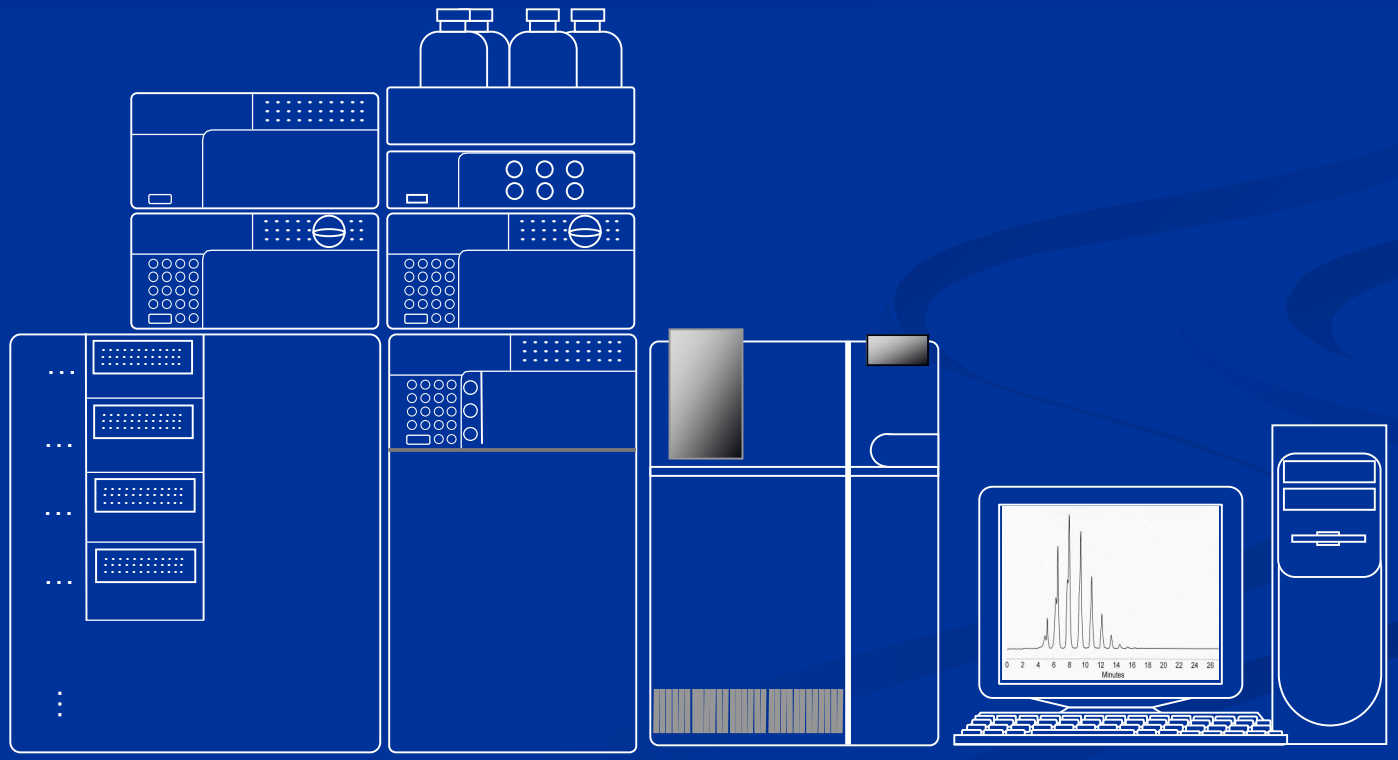
Research and Method Development

- Some “advanced” systems will include a high capacity autosampler and a mass spectrometer



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Application Questions?

Next: Software Demonstration and Prominence Hardware

Prominence Overview

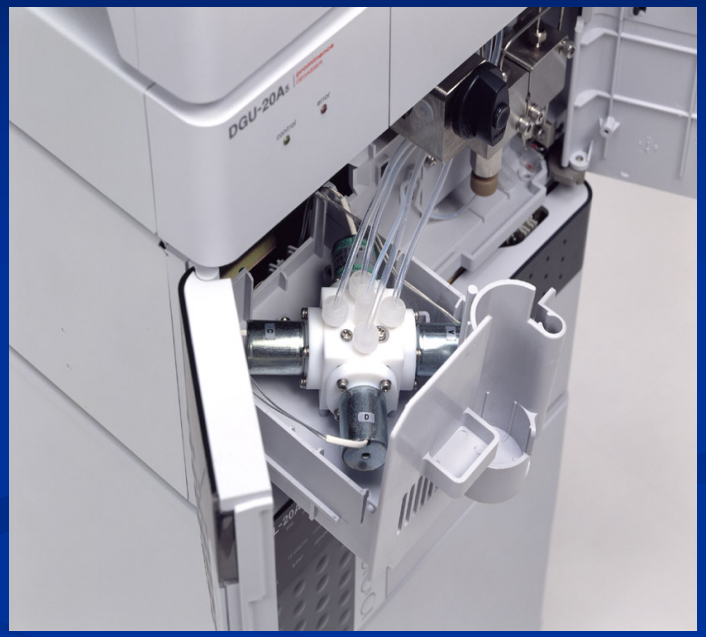
- System Controller
- Pump and Degasser
- Autosampler and Rack Changer
- Column Oven and Valves
- UV and Diode Array Detectors

CBM System Controller

- Web-based control
  - Connect to lab network or directly to computer
- Methods stored in CBM or connected computer
- Controls all components that have a fiber optic cable
  - 10A and VP Series

Standard Pump

- LC-20AT
  - 1 μL to 10 mL/minute
  - LPGE valve can be installed in the pump
  - Reduced delay volume
  - Sapphire piston and GFP seal
  - Floating piston design

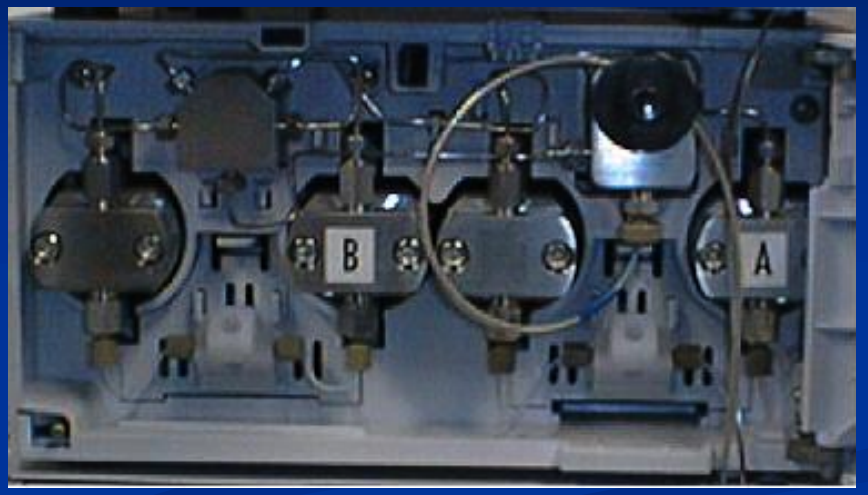


Micro-Flow Pump

- LC-20AD
  - 0.1 µL to 10.0 mL/min
  - 10 µL pistons for no pulsation
    - RID, ECD, CDD
  - Sapphire piston and GFP seal
  - Ideal for low flow rate and LCMS applications

Binary Pump

- LC-20AB
  - 2 LC-20AD in 1 box
  - Binary, space saving configuration
  - 0.1 to 10.0 mL/min
  - For gradient flow rate > 0.4 mL/minute



DGU-20A3 and A5 Degasser

- Vacuum degasser
  - Internal volume of < 400 μL
  - Teflon AF membrane for efficient O_2 removal
  - Plug into pump for power and control
  - External power supply available

Autosampler

- Two Models:
  - SIL-20A
  - SIL-20AC: 4-40C temp. control
- Enhanced Carryover Performance
- Faster Cycle Time
- Optional Active Rinsing
- Optional Rack Changer

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Rack Changer

- Two Models
  - A; ambient or C; 4-40° C, ± 6° temp. control
- 12 x 96 well MTP racks (reg. or deep well) in 4 stacks
  - Mix and match plate type between stacks
  - ~90 seconds to change plates.

Column Oven

- Forced air heating and cooling
  - CTO-20A: ambient – 85
  - CTO-20AC: (ambient -15) – 85
- Higher T.MAX for polymer and carbohydrate applications
- Linear temperature programming possible
- Integrated valve controller
- Space inside for 2 switching valves

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Switching Valves

- FCV-20AH$_2$
  - 2 Position 6 port High Pressure valve
  - Column Switching
  - Standalone control possible (front panel or Event) OR install in CTO-20A/AC
- FCV-20AH$_6$
  - 6 Position 7 port High Pressure valve
  - Column Selection
  - Standalone control possible (front panel of Event) OR install in CTO-20A/AC

UV Detector

- Extended wavelength range (190-700 nm)
- Improved Noise and Drift Specs
- Temp Controlled Flow Cell
- 2.5 AU Linear Range
- Included Hg lamp for wavelength accuracy

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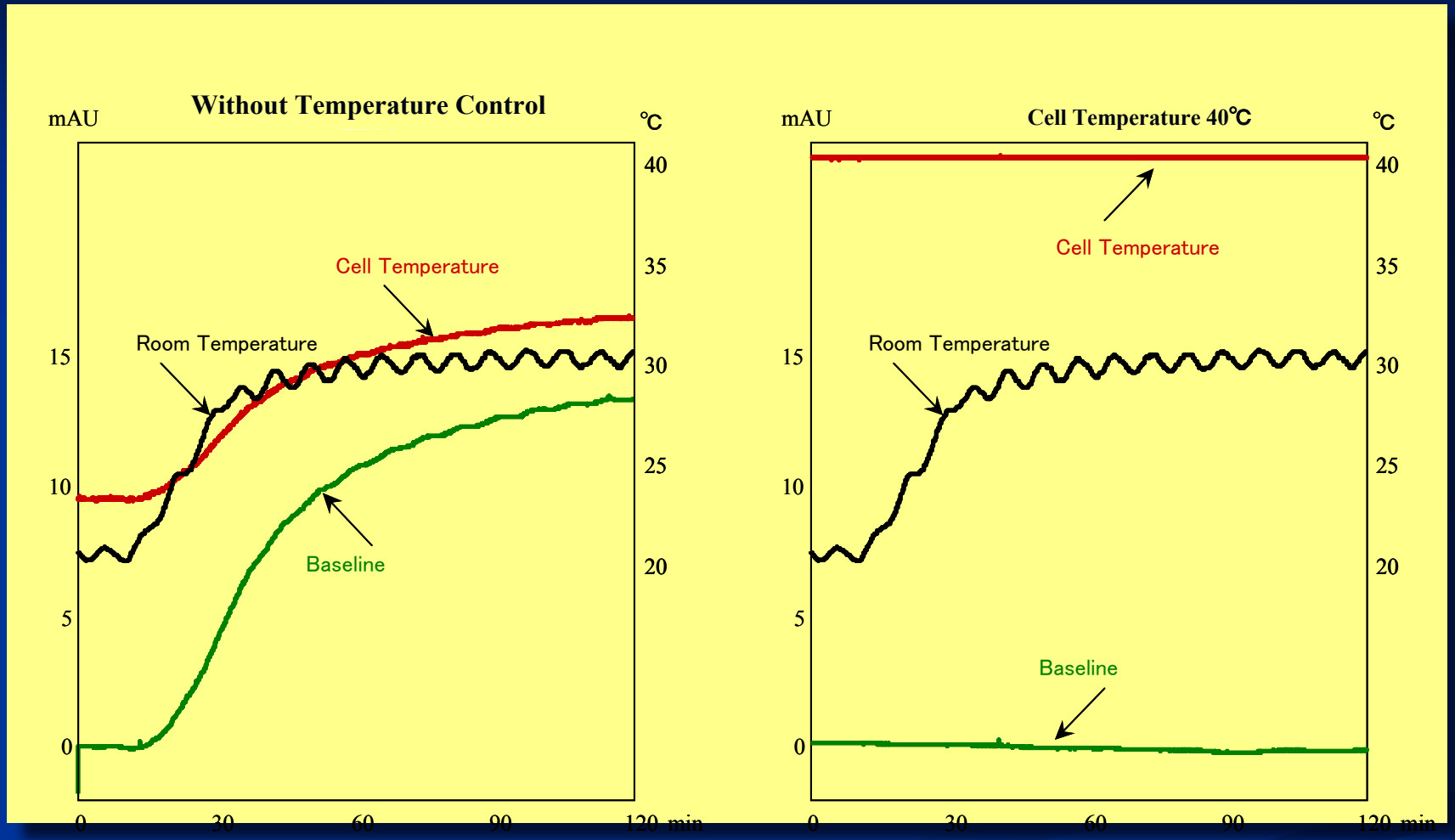
Thermostatted Flow Cell



Effect of Temperature Controlled Cell — Room Temperature raised from 20-30C

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Diode Array Detector

- World's lowest noise PDA
- World's best linearity - ≥ 2.0 AU
- Temperature Controlled Flow Cell
- Variable Slit Width
  - 8 nm (better S/N) and 1.2 nm (better resolution)
- 4 Channel Analog Board is STD
- Ethernet Communication

LC-2010 Integrated HPLC System

- Fully integrated HPLC system ideal for:
  - QA/QC environment
  - High-throughput applications
  - University teaching laboratories
- Standalone or software controlled
  - Easy to navigate control screens
  - GUI with “Wizard” assistance
  - Standard or “simple” mode

LC-2010HT Features

- Dynamic inlet valve
  - Quaternary gradient unit
- High speed autosampler
  - 4-40 C temperature control
- Column heater
- 2.5 AU detector linearity
  - Thermostatted flow cell
- Automatic power, system prep, and validation functions

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LC-2010HT Pumping System

- 5-channel degassing unit
  - 4 mL/line for solvents A-D, 2 mL/line for SIL
- Dynamic Inlet Valve
  - Electronic check valve to keep prime and minimize air bubbles
- 4 solvent proportioning valve (FCV-10ALvp style)
  - Gradient accuracy of +/- 0.5%
- Manual or automatic priming

LC-2010HT Pump Performance

- Units are pre-plumbed; users only add a column
- Instrument-to-instrument uniformity
  - 7 instruments, same column and paraben test mixture

Mean retention time, 6 reps		Methyl	Ethyl	Propyl	Butyl
S/N 005		1.693	2.217	3.245	4.457
S/N 051		1.680	2.192	3.197	4.402
S/N 054		1.677	2.185	3.177	4.387
S/N 056		1.698	2.222	3.242	4.450
S/N 058		1.690	2.208	3.222	4.415
S/N 060		1.687	2.205	3.220	4.422
S/N 062		1.670	2.173	3.155	4.363
	%RSD	0.581481	0.804374	1.04587	0.756171

LC-2010HT Autosampler

- High Capacity
  - 350 1 mL vials, 210 2 mL vials (LC-2010A), 4 microtiter plates (96 and 384 well; Std or Deep-well)
- Fast injection
  - 15 second injection, ~30 second cycle time
- Reproducibility < 0.3% RSD specification
  - Typical value: ~0.10%
- Low carryover: < 0.01% (napthalene analysis)
  - NEW Pt coated needle, PEEK rotor and PEEK needle seal to further reduce carryover

LC-2010HT Autosampler Performance

- Injection Reproducibilty
  - Method: Isocratic premixed 60:40 MeOH:H_2O
  - Sample: Paraben test mix; 1, 5, 10, 25, and 50 µL injections, 10 reps each

LC-2010A	1 µL %RSD	5 µL %RSD	10 µL %RSD	25 µL %RSD	50 µL %RSD
methyl paraben	0.295	0.0549	0.0393	0.0685	0.0425
ethyl paraben	0.228	0.0705	0.0385	0.0370	0.0560
propyl paraben	0.327	0.0533	0.0509	0.0233	0.0463
butyl paraben	0.285	0.0773	0.0336	0.0376	0.0439

LC-2010C	1 µL %RSD	5 µL %RSD	10 µL %RSD	25 µL %RSD	50 µL %RSD
methyl paraben	0.283	0.0562	0.0392	0.0223	0.0515
ethyl paraben	0.265	0.0533	0.0335	0.0325	0.0473
propyl paraben	0.246	0.0511	0.0511	0.0427	0.0405
butyl paraben	0.265	0.0310	0.0204	0.0206	0.1210

LC-2010HT Autosampler Performance

- Injector cycle time is crucial for high-throughput and mass spec. applications
- The LC-2010HT can inject in ~15 seconds
- Actual time, from pressing RUN to injection

	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6
LC-2010A	15.58	15.49	15.24	15.52	15.43	15.64

LC-2010HT Autosampler Performance

- Injection linearity
  - Paraben test mix: 1, 5, 10, 25, 50 µL injections
  - 10 repetitions per level

LC-2010A	R^2
methyl paraben	0.999976
ethyl paraben	0.999982
propyl paraben	0.999987
butyl paraben	0.999989

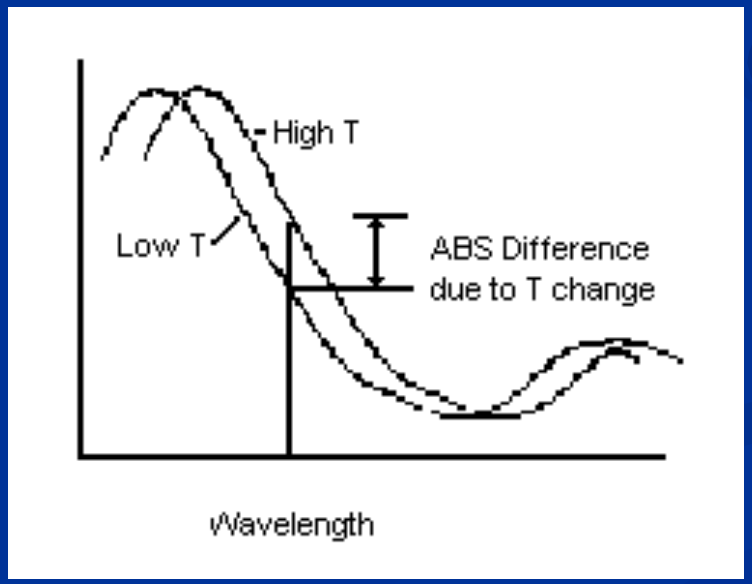
LC-2010C	R^2
methyl paraben	0.999997
ethyl paraben	0.999994
propyl paraben	0.999995
butyl paraben	0.999994

LC-2010HT Column Oven

- Block style that heats and cools column
  - Setting range of (Ambient - 15) to 60 C
- Adjustable aluminum blocks for extra contact points with column
- Solvent preheater: 4 or 9 µL
- Mixer in direct contact with heating block
  - Mixer volume is 240 µL

LC-2010HT Detector

- 2.5 AU linearity spec
- Built in Hg lamp for wavelength calibration
- Thermostatted flow cell: 40 and 50 C settings
  - Prevents change in absorbance due to refractive index change with temperature variations



LC-2010HT Detector Performance

- Linear to 2.5 AU
- Prednisone: 5 concentration levels
  - 10 µL injections, 5 reps at each level
  - 60:40 MeOH:H_2O, 4.6x100mm C18 column

LC-2010C S/N 002				
Level 1	0.045 mg/mL	144 mAU		
Level 2	0.090 mg/mL	294 mAU		
Level 3	0.180 mg/mL	600 mAU		
Level 4	0.360 mg/mL	1215 mAU		
Level 5	0.720 mg/mL	2550 mAU	R^2 = 0.999800	
LC-2010C S/N 003				
Level 1	0.045 mg/mL	141 mAU		
Level 2	0.090 mg/mL	288 mAU		
Level 3	0.180 mg/mL	585 mAU		
Level 4	0.360 mg/mL	1190 mAU		
Level 5	0.720 mg/mL	2530 mAU	R^2 = 0.999688	

Additional LC-2010HT Features

- Automatic power on/off
- System Prep – for running samples with different solvents
- Automatic system validation
- Individual component validation
- Status screen – mobile phase calculation
- Maintenance/parts replacement