HPLC Method Development

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HPLC Method Development

I. Method Development Strategy

II. Selecting a Detector

III. Selecting the Separation Mode

IV. Mobile Phase and pH

V. Optimization Based on the Resolution Equation

Overview of Method Development Strategy

- Define method goals
- Establish sample prep procedure
- Select detector
- Select mode of separation
  - Column and mobile phase
- Perform preliminary separations
- Optimize conditions
- Calibrate and validate

Define Method Goals

- What is known about the sample?
- What level of detection is required?
- Are standards available?
- How fast does the analysis need to be?
- How much resolution is required?

What is Known About the Sample?

- Chemical structure(s)
- Acidic/Basic, pKa
- Molecular weight
- Stability (light and solvents)
- Solubility
- Concentration
- Matrix

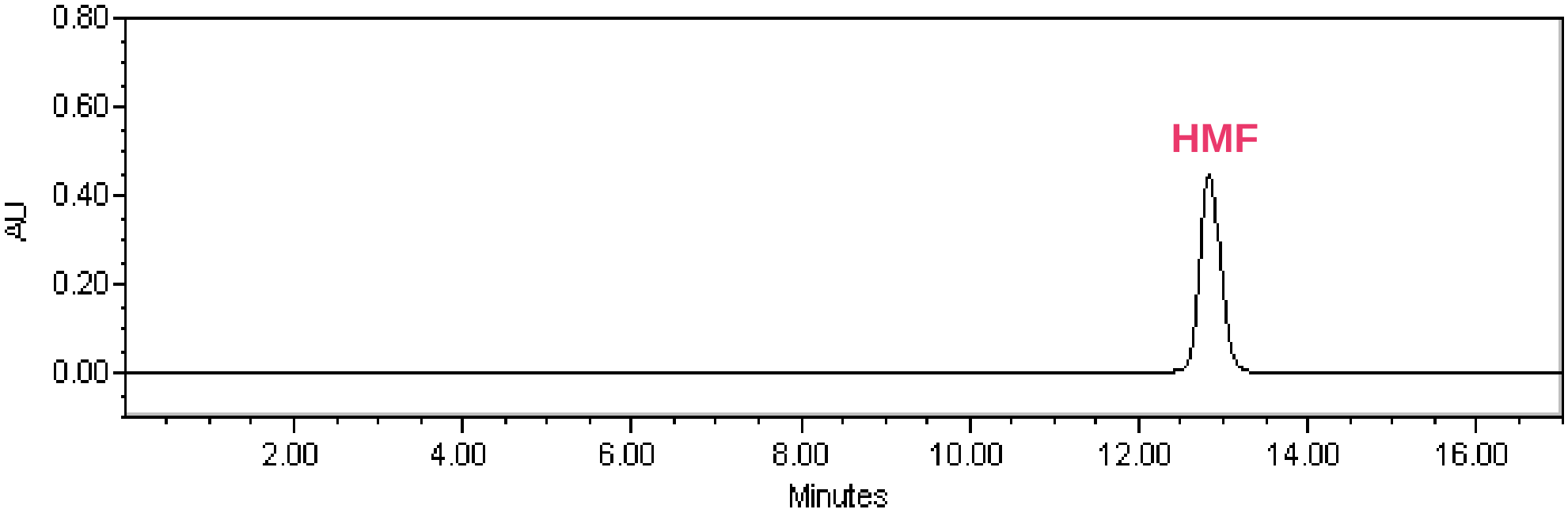
Sample Prep Options

- Filtration
- Centrifugation
- Solvent extraction
- Solid Phase Extraction (SPE)
- Supercritical Fluid Extraction (SFE)
- Preparative Chromatography
- Column Switching
- Derivatization

Sample Prep Guidelines

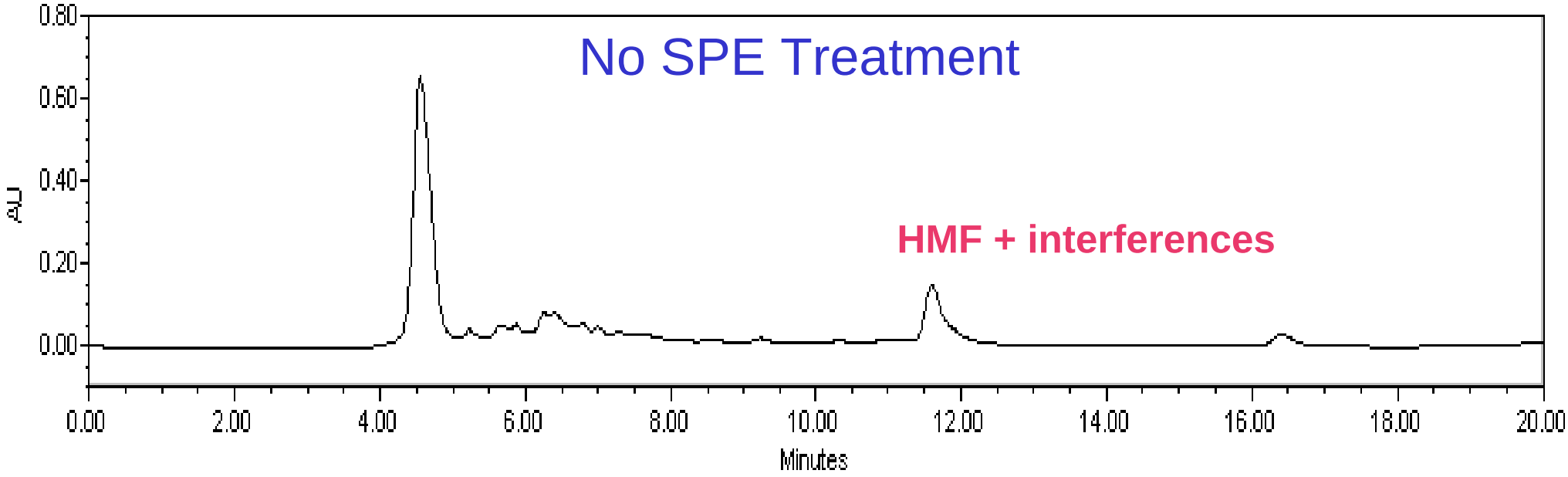
- Minimum sample purity required:
  - Free of particulates
  - Completely soluble in mobile phase
- Start with minimal sample prep
  - Filtration is often sufficient
- Additional sample prep needs may be identified during preliminary separations

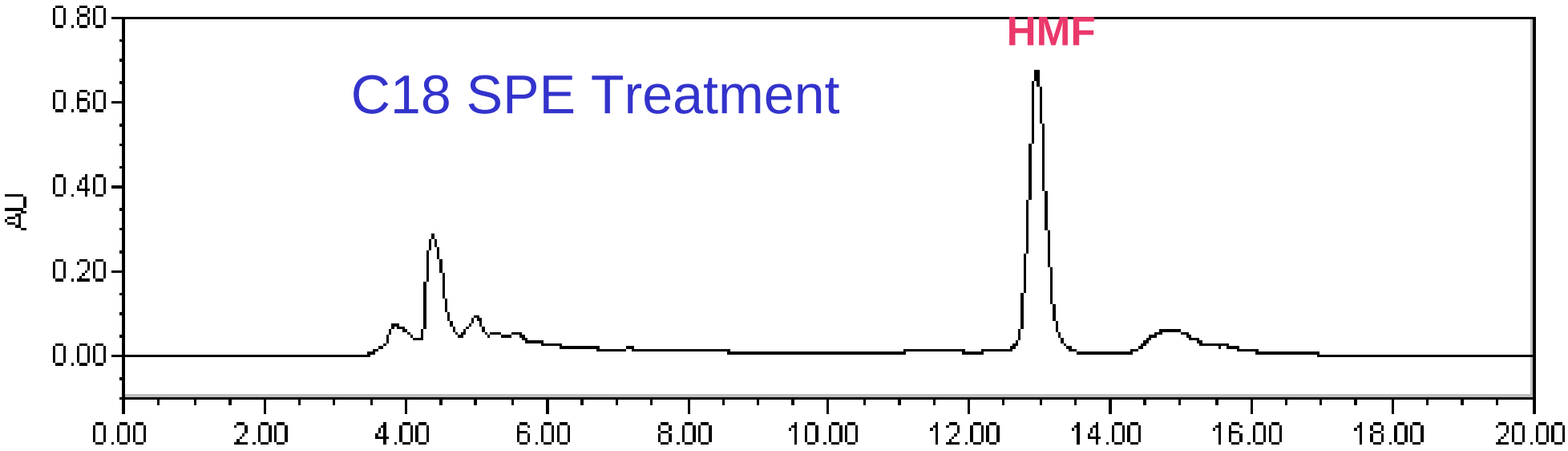
Sample Prep: HMF Standard by HPLC



Column: Ultra C18 (Restek Corp.), 250 mm x 4.6 mm, 5 µm
Mobile Phase A: 90:10 water:methanol, 10 mM ammonium formate
Mobile Phase B: 10:90 water:methanol, 10 mM ammonium formate
Gradient: 0-5 min at 100% A, to 100% B at 10 min, 10 min. hold
Flow: 0.5 mL/min.
Temperature: ambient
Detector: UV @ 280 nm
Injection Volume: 10 µL

Sample Prep: HMF in Grape Juice by HPLC





Detector Selection: Types

- Ultraviolet/Visible Absorbance (UV/Vis)
- Mass Spectrometer (MS)
- Refractive Index (RI)
- Evaporative Light Scattering (ELS)
- Fluorescence (FL)
- Electrochemical (EC)

Detector Selection

- Selection is Based on:
  - Chemical nature of analytes and potential interferences
  - Limit of detection required
  - Availability and/or cost of detector

Detector Selection: UV/Vis

- Requirement: analyte must absorb more light than sample matrix at some wavelength
- Most widely used
- Most compounds absorb at low UV
- Diode Array Detector (DAD) can monitor multiple wavelengths simultaneously

Detector Selection: UV/Vis

Chromophore	Formula	λmax (nm)
Amine	$-NH_2$	195
Ethylene	-C=C-	190
Ketone	RR'C=O	195
Ester	ROC=O	205
Aldehyde	RHC=O	210
Carboxyl	COOH	200-210
Nitro	NO_2	310
Phenyl	$-C_6H_5$	202,255
Naphthyl		220,275

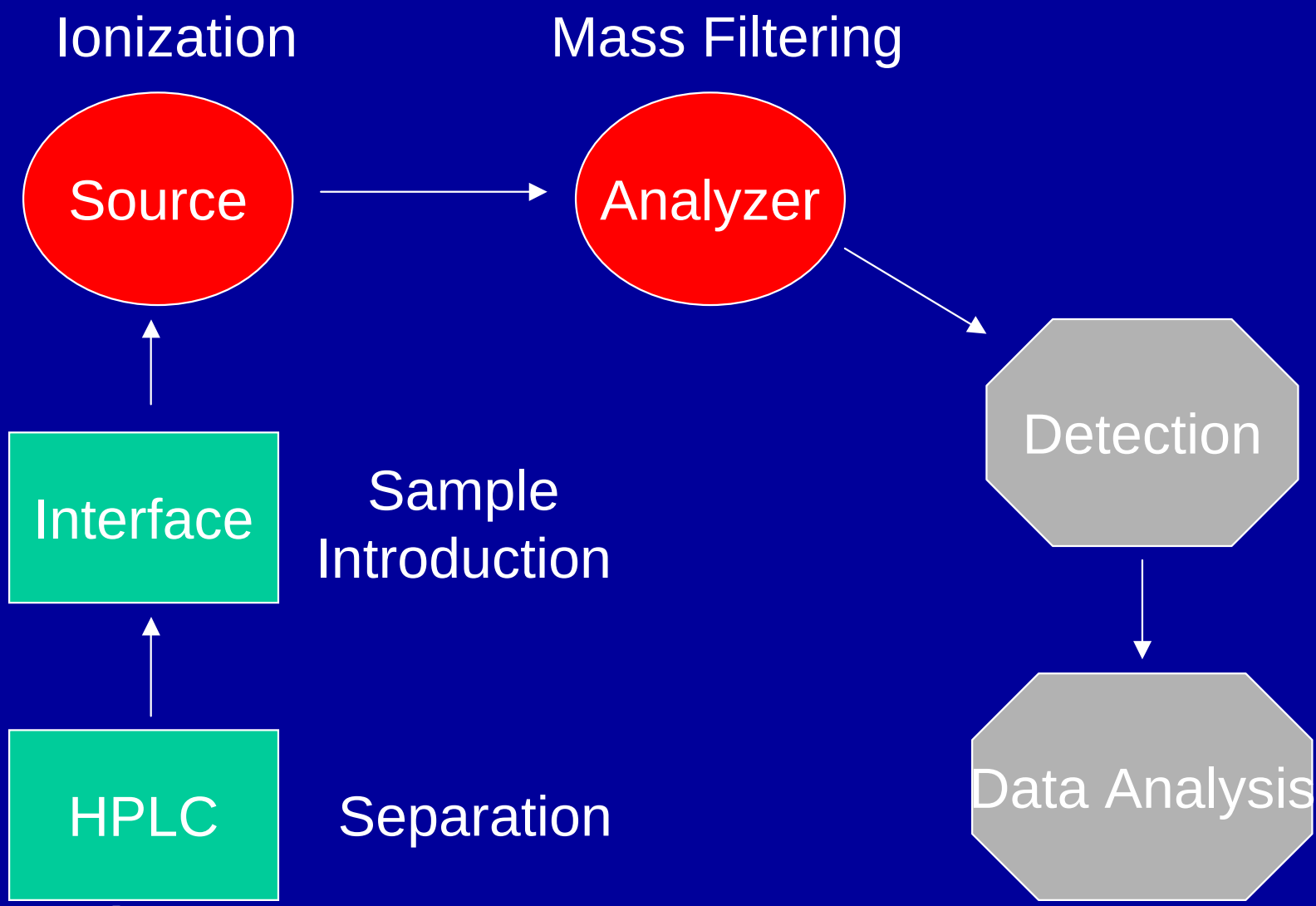
Detector Selection: UV/Vis

- Choose detection wavelength that maximizes sensitivity and specificity
- Solvents used may cause slight shifts in UV_{max} from published values (2-5 nm)
  - Check absorbance of analyte in mobile phase
- Mobile phase solvents have UV cutoff points
  - Operating below cutoff point will:
    - Reduce sensitivity
    - Add to baseline noise

Detector Selection: Mass Spectrometer

- Requirement: analyte must be ionizable
- Rapidly growing in popularity
- Positive identification of analyte
- Can discriminate between co-eluting peaks in selected ion mode
  - Reduces resolution required
- For best sensitivity, work at pH where analytes are ionized
  - Neutral to basic pH (7-9) for acids
  - Acidic pH (3-4) for bases

Detector Selection: Mass Spectrometer



Detector Selection: Refractive Index

- Monitors difference in the refractive index of the sample cell vs. the reference cell
- Non-selective
- Concentration dependent
- Sensitivity is typically 100x-1000x less than a UV/Vis detector
- Cannot be used with gradients (without special modifications)

Detector Selection: Light Scattering ELSD

- Detector is mass dependent and non-selective
- Ideal for:
  - High molecular weight compounds
  - Sugars and less volatile acids
- Amount of light scattering is related to the molecular mass of the analyte
- Can be used with gradient systems
- Solvents should be volatile for best results

Advances in Instrumentation
Light Scattering Detectors

3 distinct processes:

- Nebulization of the mobile phase
- Evaporation of the mobile phase
- Light scattering by analyte particles

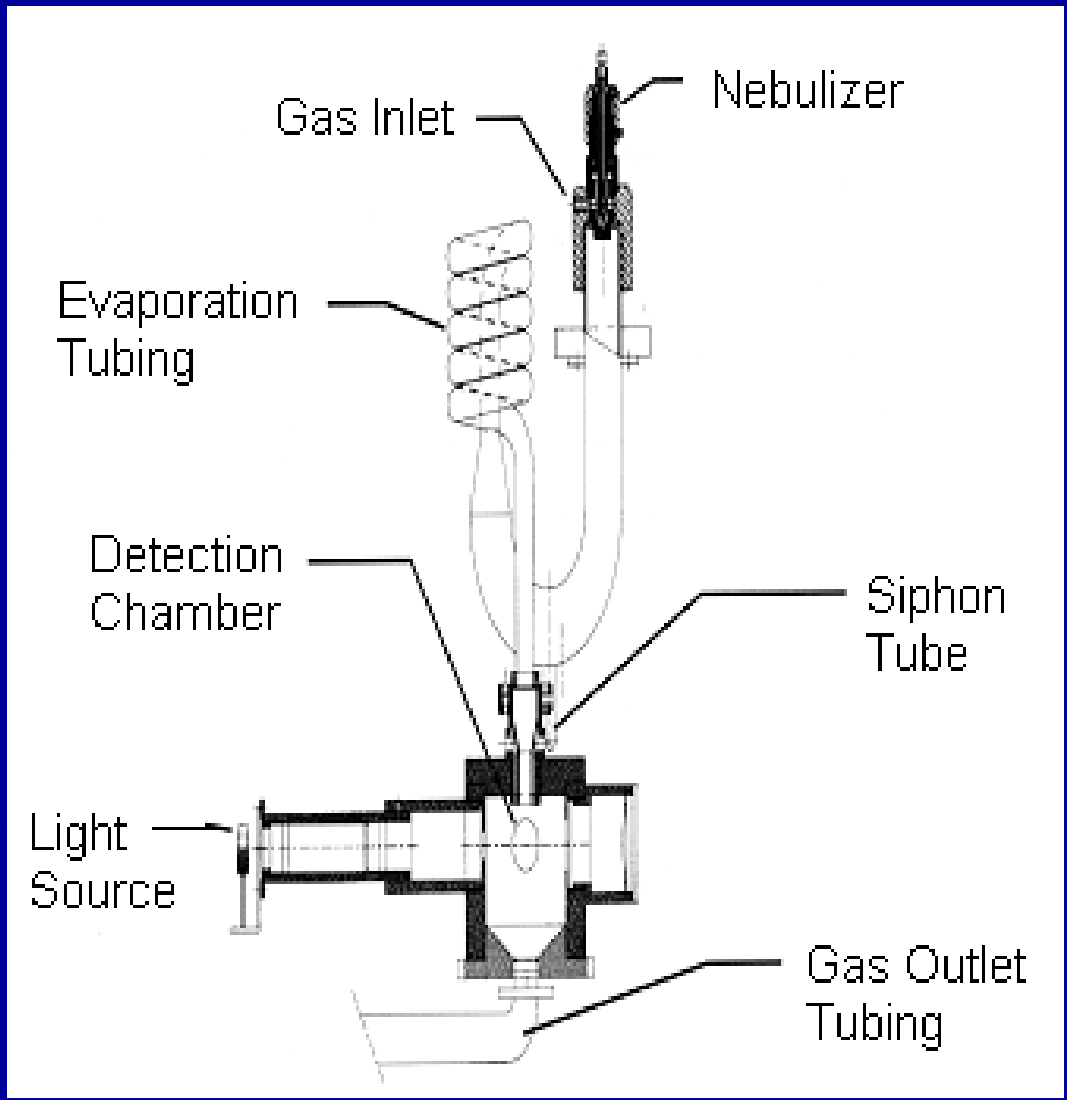


Diagram courtesy of ESA, Inc.

Detector Selection: Fluorescence

- Analyte must fluoresce
- Excite at one wavelength, measure the emission at a longer wavelength
- Up to 1000x more sensitive than UV/Vis
- High specificity
- Concentration dependent
- Operation similar to a UV/Vis detector

Detector Selection: Electrochemical

- Requirement: Analytes can be oxidized or reduced by an electrical current
- More sensitive than fluorescence
- Not as selective as fluorescence (typically)
- Not compatible with gradient elution

Detector Selection: Approximate LODs

- EC 10^{-12}
- MS 10^{-11}
- FL 10^{-11}
- UV 10^{-10}
- RI 10^{-7}
- ELS 10^{-7}

Selecting the Mode of Separation

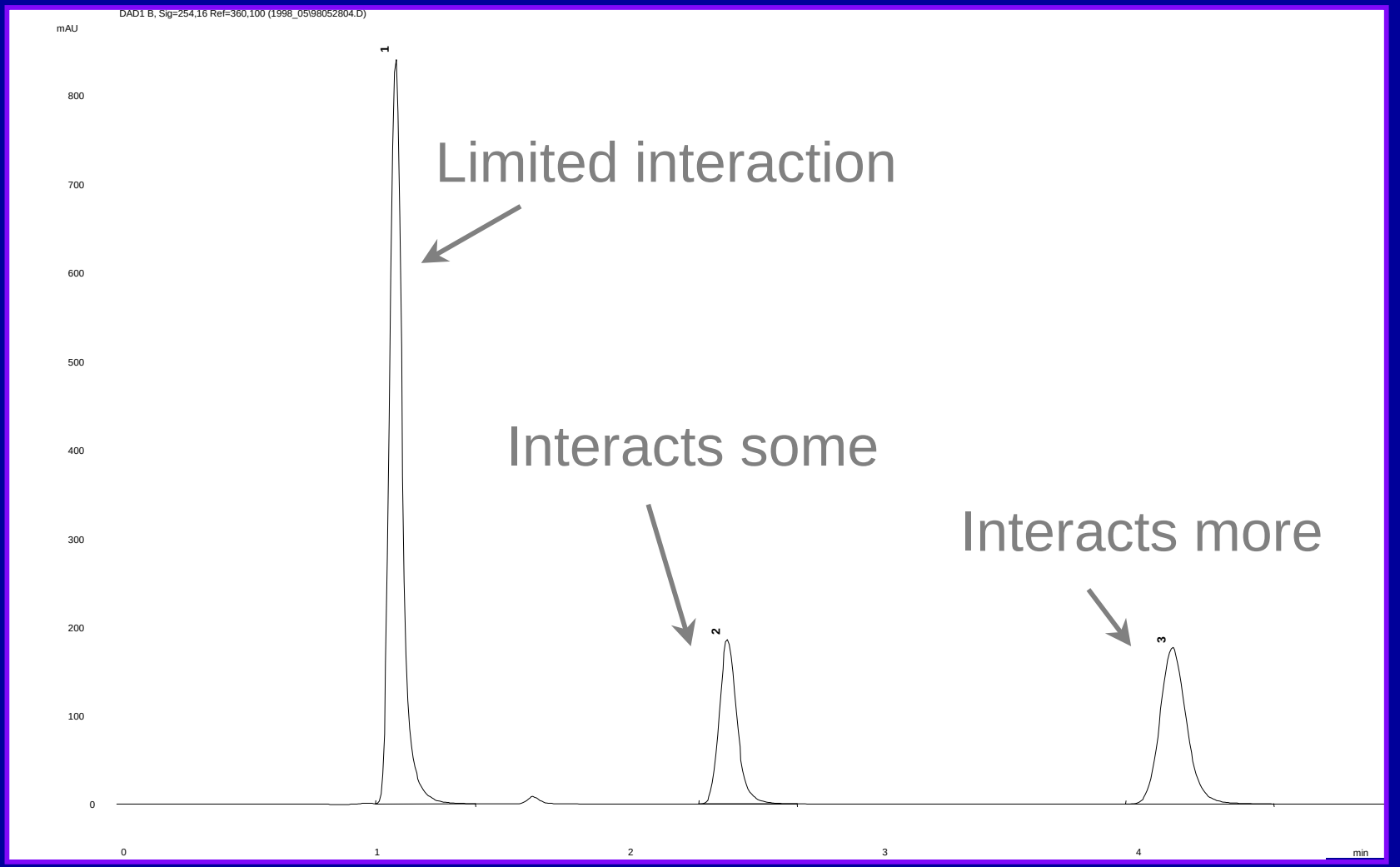
- Sample solubility
- How do analytes of interest differ from other compounds in sample?
- Reversed phase is the most frequently used mode

Mode Selection : Reversed Phase

- Mobile phase is polar
- Stationary phase is less polar
- Major distinction between analytes is their hydrophobicity
- The sample should be soluble in water or a polar organic solvent (i.e. methanol)
- Examples are C18 (ODS), C8 (Octyl), Phenyl, Butyl, and Methyl

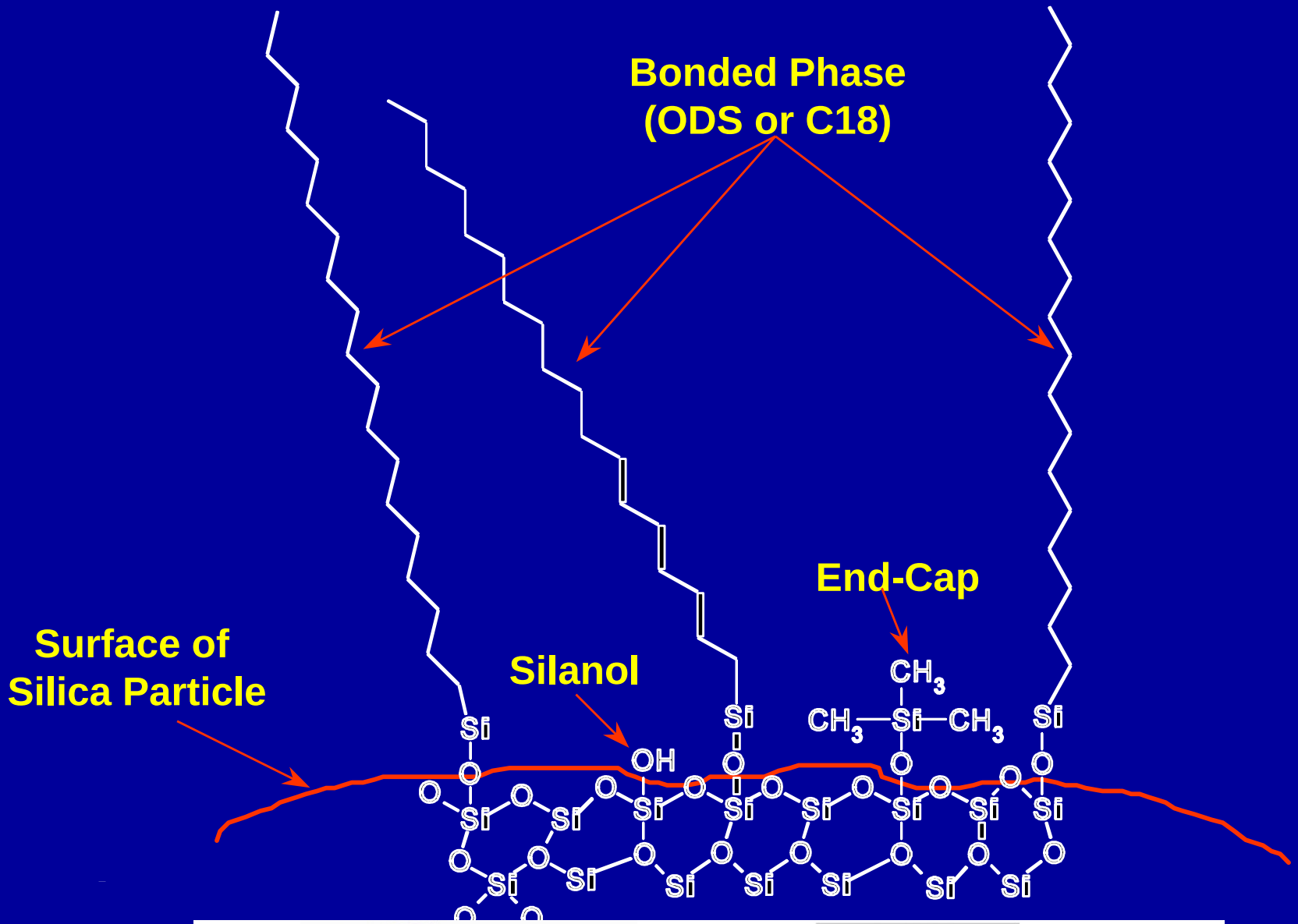
Mode Selection : Reversed Phase

Nonionic Compounds



Hydrophilic → Hydrophobic

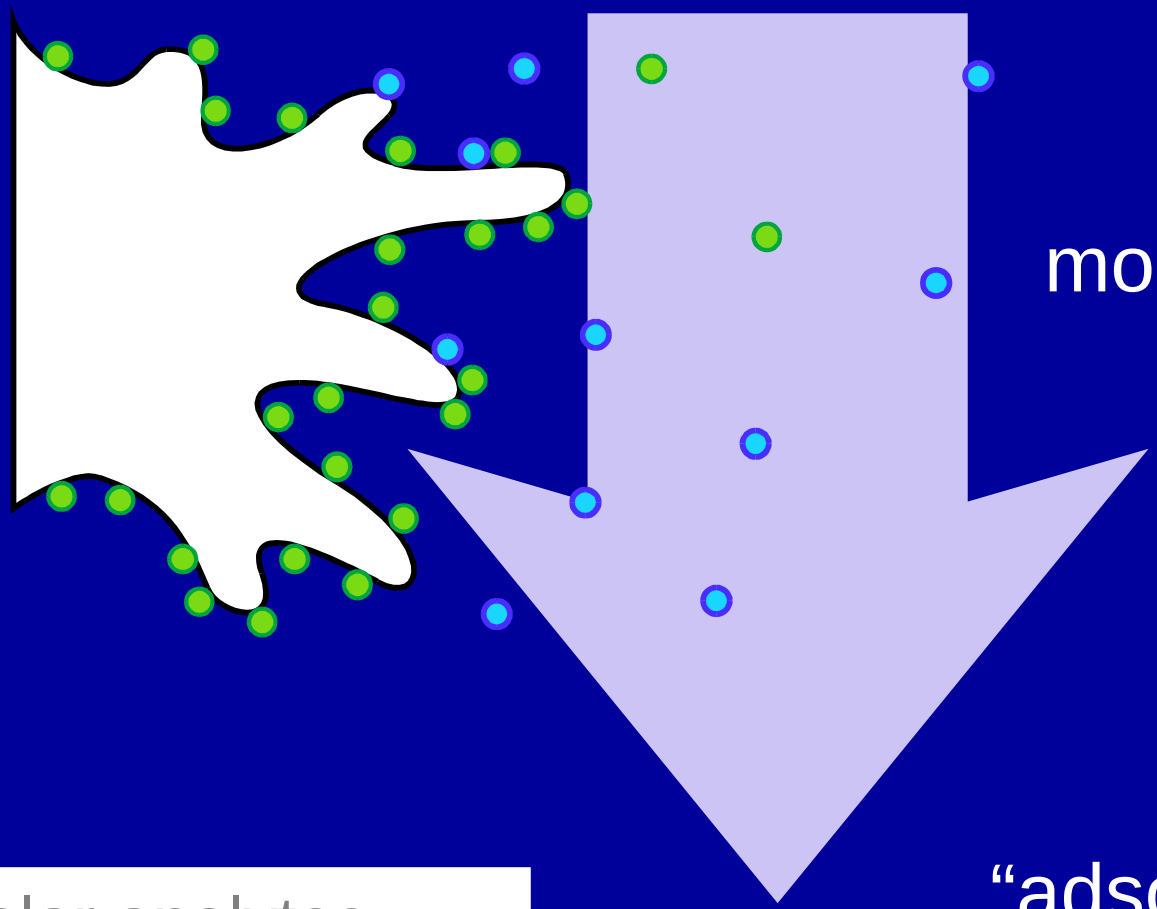
Reversed Phase: The Bonded Phase Surface



Mode Selection : Normal Phase

- Mobile phase is non-polar while stationary phase is more polar
- When a major distinction between analytes is NOT their hydrophobicity
- Sample should be soluble in a hydrophobic solvent such as hexane
- Mobile phase is a weak to moderate solvent for the sample
- Examples are Silica, Cyano, Amino and Diol

Normal Phase Mode of Separation

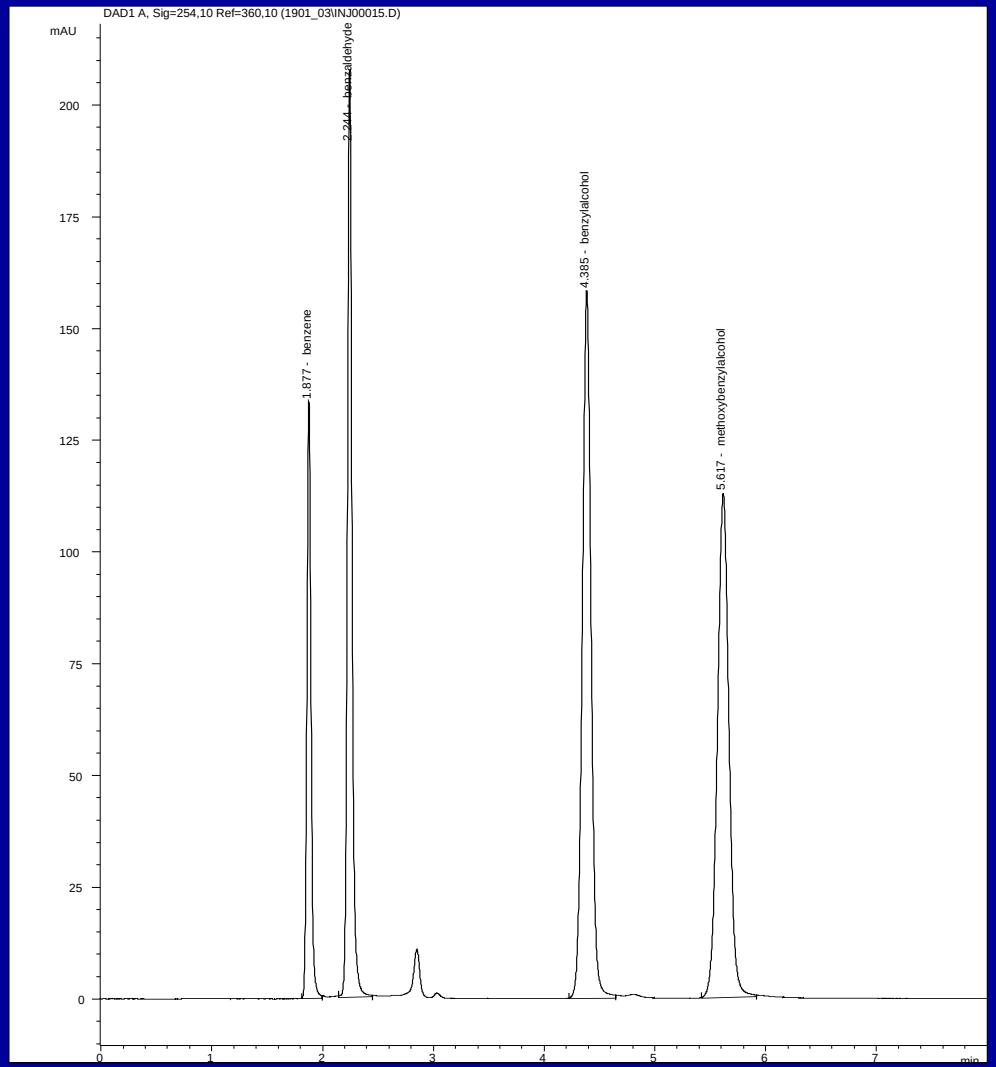


- Polar analytes
- Less polar analytes

"adsorption" mechanism

Normal Phase Test Mix

Pinnacle II Silica Column



Peak list:

1. benzene
2. benzylaldehyde
3. benzylalcohol
4. methoxybenzylalcohol

Column: Pinnacle II Si,
150x4.6mm, 5µm

Mobile phase: 96% hexane:4% IPA

Flow: 1.0 ml/min

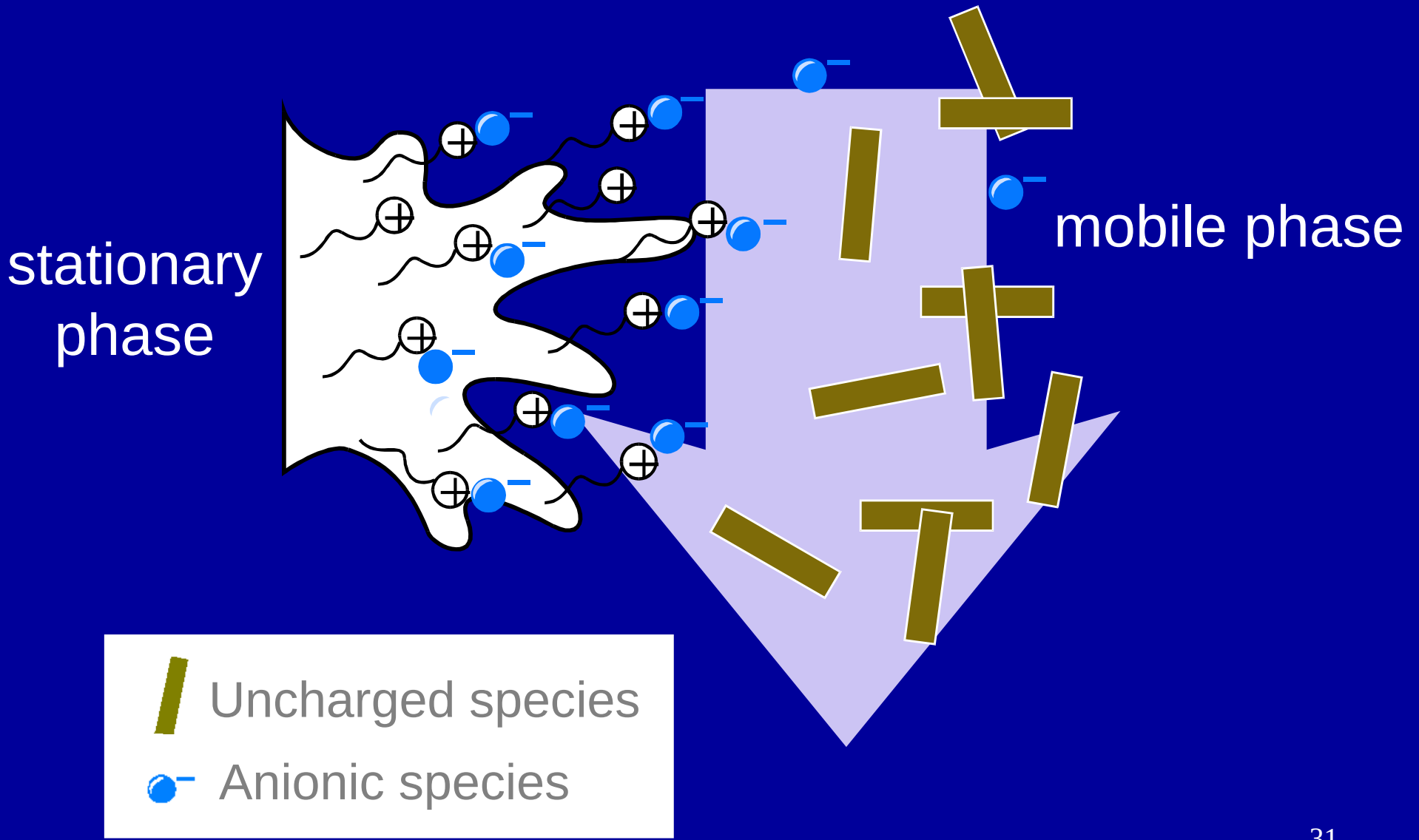
Temp.: ambient

Det.: UV @ 254

Mode Selection : Ion Exchange

- When analytes are ionic or potentially ionic
- Mobile phase is typically an aqueous buffer
- Mobile phase strength is a function of ionic strength
- pH is critical
- SAX is Strong Anion Exchange (WAX = Weak)
- SCX is Strong Cation Exchange (WCX = Weak)
- Examples
  - Inorganic Cations and Anions, Organic Acids and Bases, Amino Acids, Nucleotides, Catecholamines, Peptides, Antibiotics

Mode Selection : Ion Exchange



Mode Selection: Reversed Phase Ion-Pair

- When analytes are ionic or potentially ionic
- Mobile phase is composed of a buffer, an ion-pair reagent and a polar organic solvent
- Typical ion-pair reagents include
  - Alkyl sulfonates (heptane sulfonic acid, octane sulfonic acid) for bases
  - Quaternary amines (tetrabutylammonium chloride) for acids

Mode Selection: Size Exclusion

- Major distinction between the analytes in the mixture is their hydrodynamic volume
- Generally for molecular weights > 2000
- Want to avoid partitioning
- The mobile phase should be a strong solvent for the sample
- Aqueous SEC is called Gel Filtration
  - Proteins and other biomolecules
- Organic SEC is called Gel Permeation (GPC)
  - Polymers

Why Is Reversed Phase the Most Popular Mode?

- Large proportion of analytes are water soluble
- Wide range of stable stationary phases available
  - used to alter retention and selectivity
- Simple mobile phases work for many applications (i.e. water:acetonitrile)
- Selectivity can be altered by changing the mobile phase

General Rules for Mobile Phase Selection

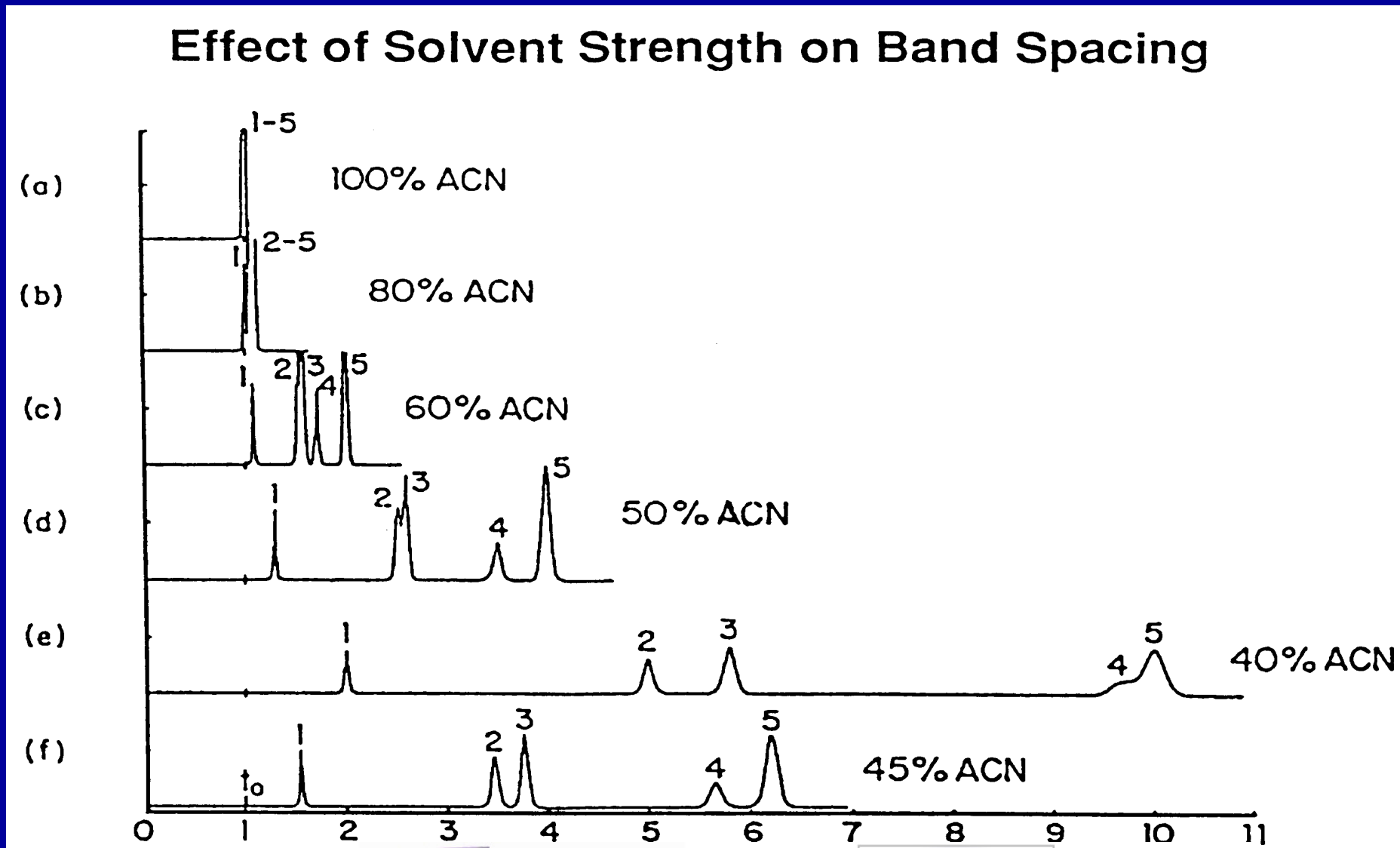
- In partition chromatography, the mobile phase should be a moderate to poor solvent for the samples
  - Produce a capacity factor of 1 to 10 (2 to 5)
- For ion exchange and size exclusion the mobile phase should be a strong solvent for the sample
- The use of *additives or modifiers* can enhance a separation
  - Improving peak shape
  - Altering selectivity

Mobile Phase Selection: Organic Solvent

- Water miscible
- Low viscosity
- Low UV cut-off
- Unreactive
- Most commonly used:
  - Acetonitrile
  - Methanol
  - Tetrahydrofuran (THF)

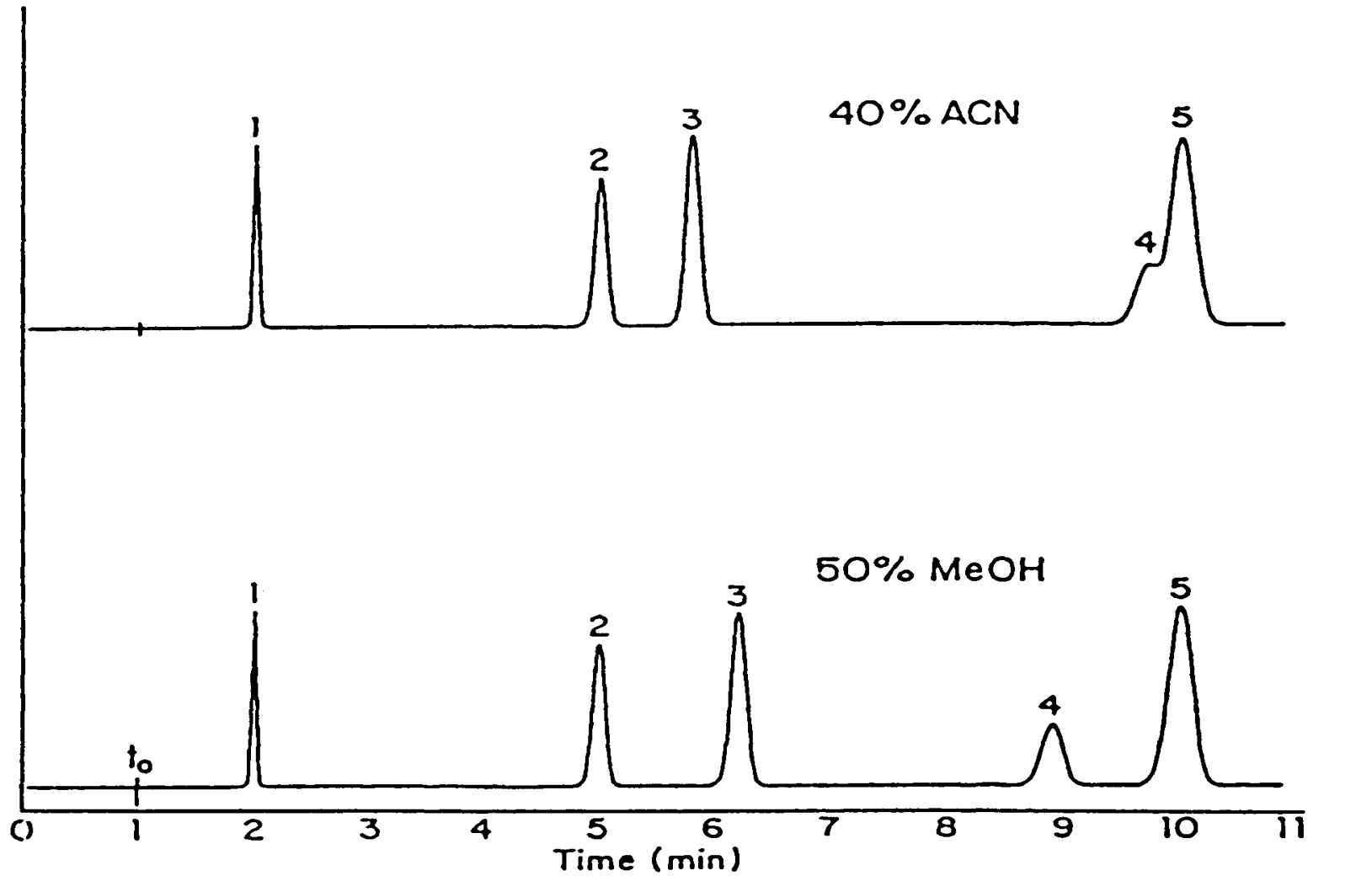
Mobile Phase Selection

Adjusting Retention with %B



Mobile Phase Selection

Changing the Selectivity



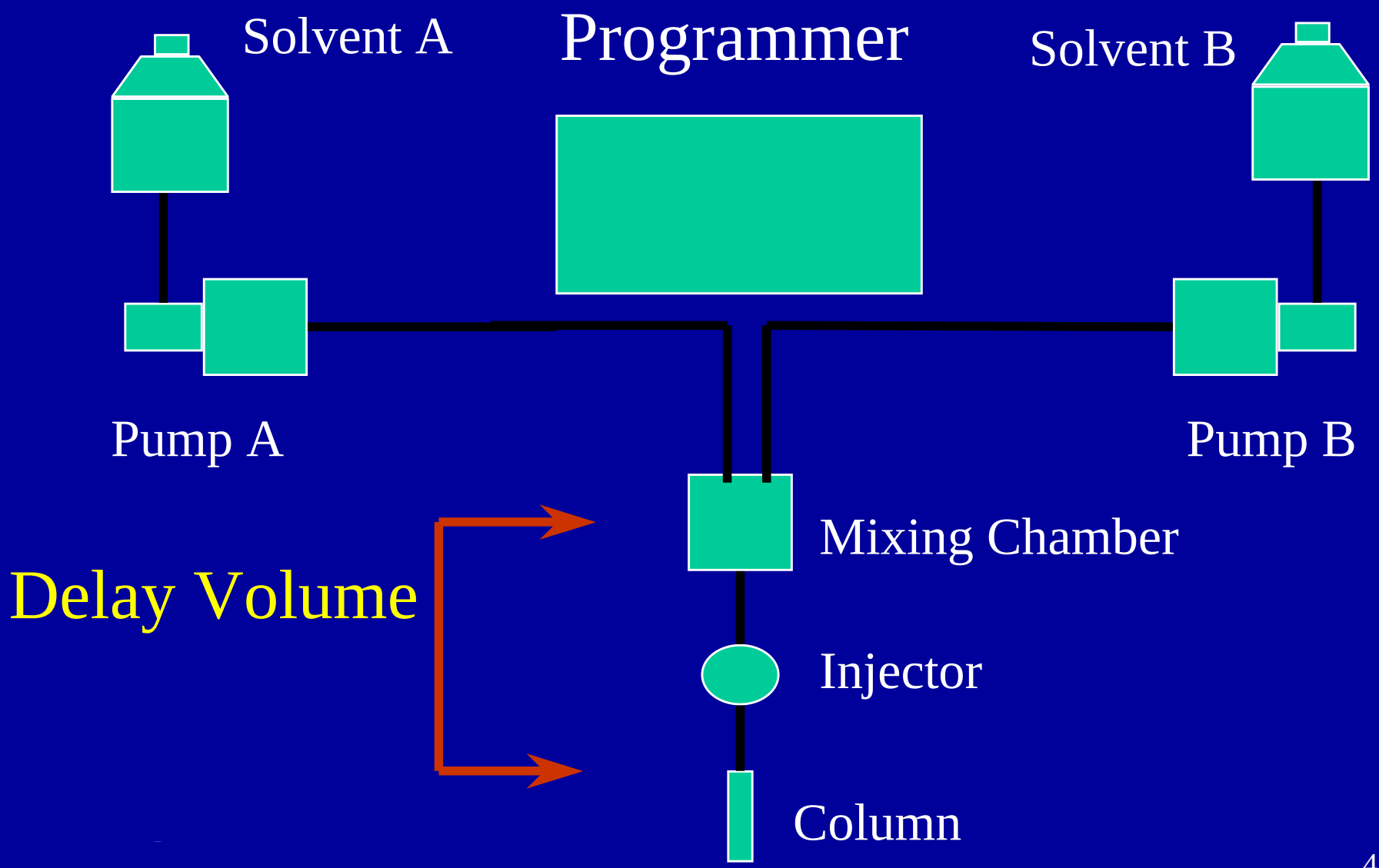
What if a single mobile phase (isocratic) will not elute all analytes in the desired k' range?

- Use gradient elution
- Mobile phase strength changes over time
- Weak mobile phase early in the gradient
  - k'>2 for weakly retained analytes
- Strong mobile phase later in the gradient
  - k'<10 for strongly retained analytes
- Initial scouting run
  - Use to estimate %organic for an appropriate elution
  - Elutes all strongly-retained compounds

Disadvantages Of Gradient Elution

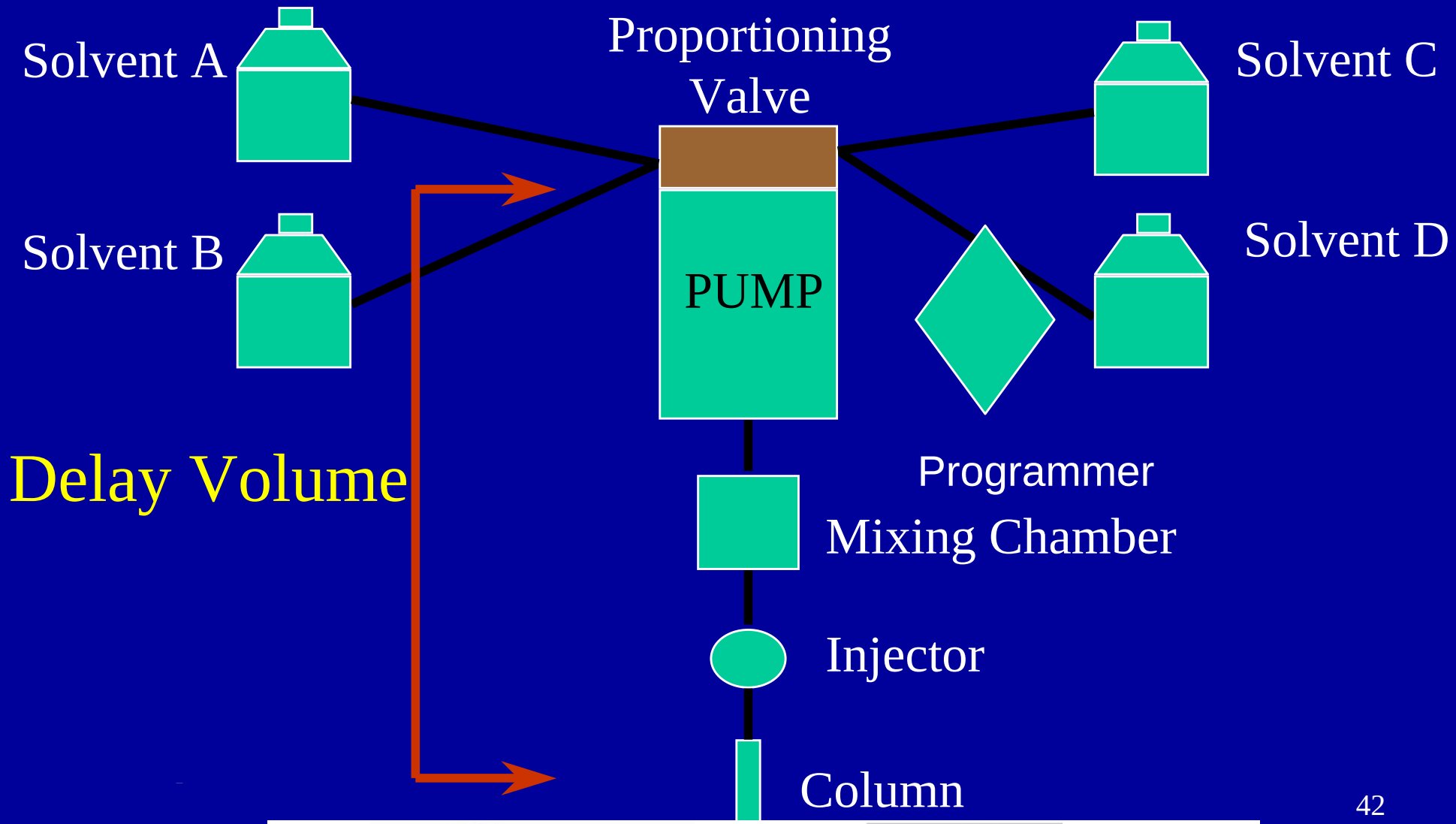
- Column re-equilibration required after every analysis
- Requires a pump with at least two-solvent capability
- Not compatible with some forms of detection (RI, EC)
- More variables to control for reproducibility
- Delay volume becomes important
  - Volume of mobile phase contained in the HPLC system between pump(s) and column

High Pressure Gradient System

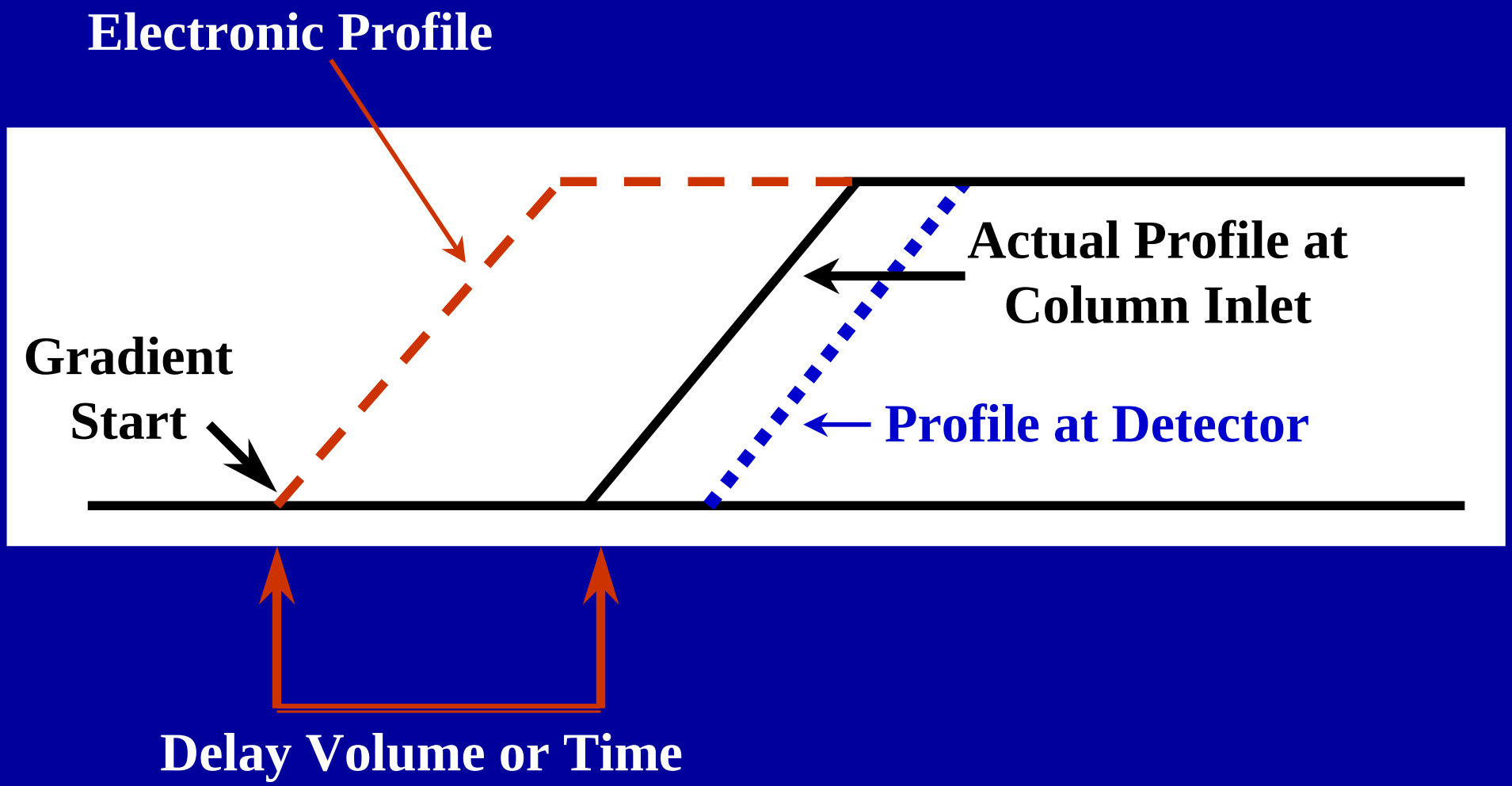


Low Pressure Gradient System

Quaternary



Gradient Profile



Measure using (A) methanol or water, (B) A + 1% acetone
Step gradient from 100%A to 100%B, UV @ 265nm

Gradient Variables

- Solvent selection and mobile phase composition
- Gradient shape
- Gradient steepness
- Duration and position of isocratic conditions
- Pressure and flow

Tips for a Successful Gradient Run

- Keep it as simple as possible
- Be aware that delay volumes will vary from instrument to instrument
- Make sure post run equilibration time is adequate to return column to initial conditions
- Pre-mix mobile phase modifiers
- Pre-mix solvents with poor miscibility
- Avoid ion-pair gradients

Mobile Phase Selection

pKa and Mobile Phase pH

- pH is an important consideration in method development
- At a pH close to the pKa, peak distortion results
- Partial dissociation of a weak acid or base into its conjugate form

$$HA \rightleftharpoons H^+ + A^-$$

Dissociation of a weak acid

$$pH = pK + \log \frac{[A^-]}{[HA]}$$

Henderson-Hasselbalch Equation

Buffers for Reverse Phase HPLC

pH Range	Buffer	UV cutoff (nm)
1.1 - 3.1 6.2 - 8.2 11.3 - 13.3	phosphate	210
2.1 - 4.1 3.7 - 5.7 4.4 - 6.4	citrate	250
3.8 - 5.8	acetate	230
7.3 - 9.3	tris(hydroxymethyl) aminomethane	220
8.2 - 10.2	borate	210

Mobile Phase Selection

Incorrect pH for Tetracyclines

- The pKa for tetracylines is ~3.3
- At pH 3, the form of the analyte is in a ratio of 2:1 weak acid to conjugate base

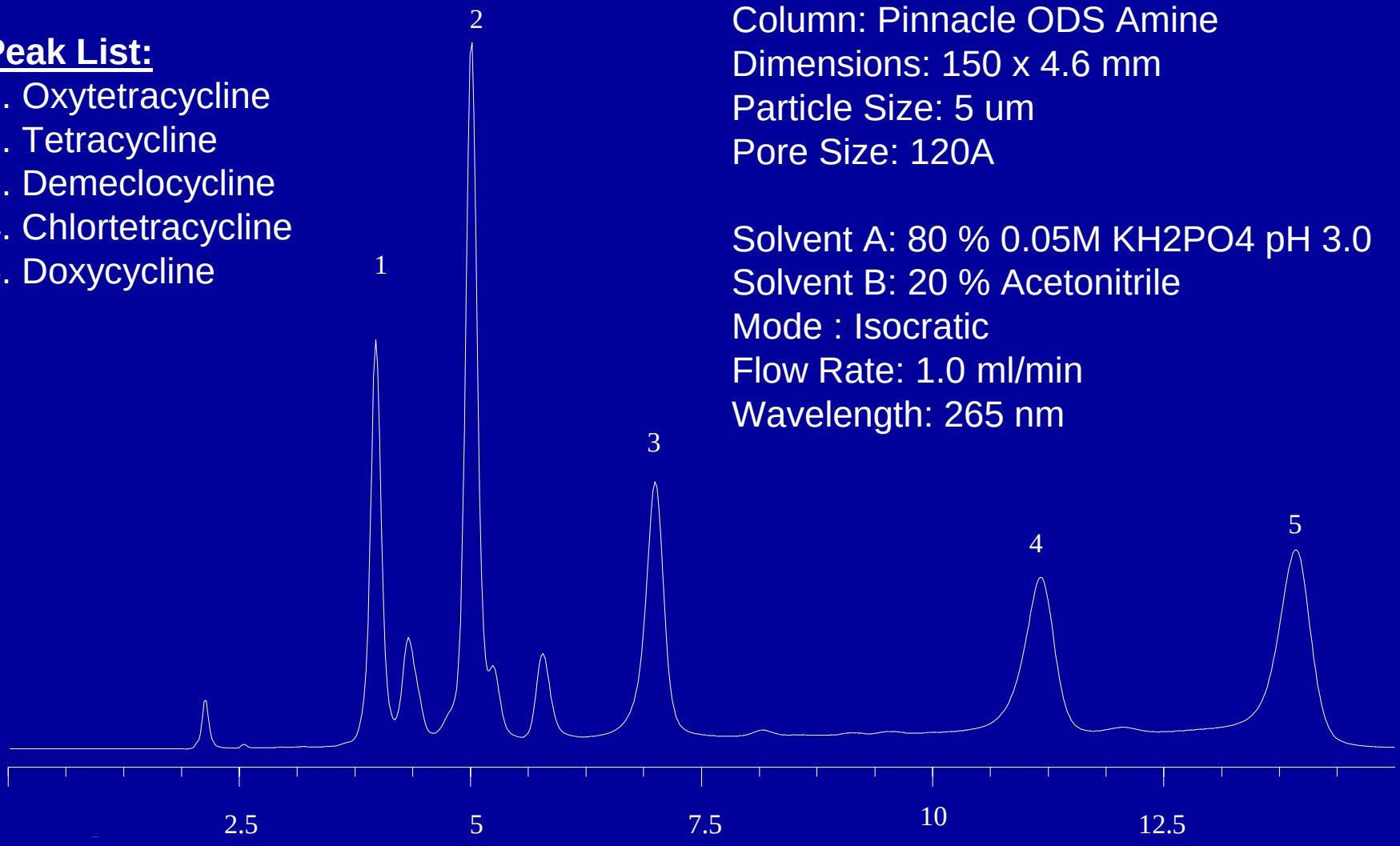
$$\log \frac{[A^-]}{[HA]} = pH - pK$$

$$\log \frac{[A^-]}{[HA]} = 3 - 3.3 = -0.3$$

$$\frac{[A^-]}{[HA]} = 0.5$$

Tetracyclines at pH 3

Peak List:
1. Oxytetracycline
2. Tetracycline
3. Demeclocycline
4. Chlortetracycline
5. Doxycycline



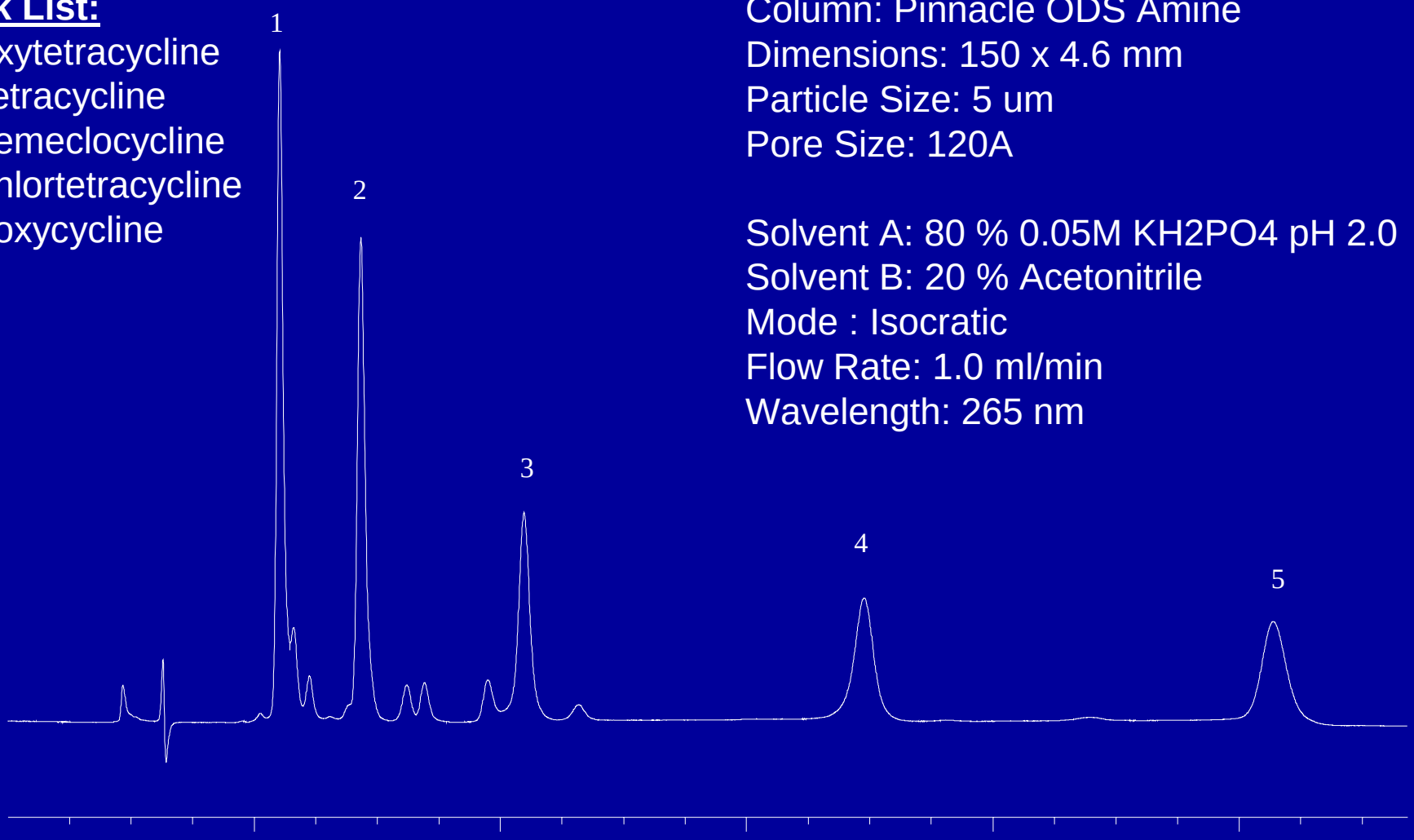
Conditions:
Column: Pinnacle ODS Amine
Dimensions: 150 x 4.6 mm
Particle Size: 5 um
Pore Size: 120A

Solvent A: 80 % 0.05M KH2PO4 pH 3.0
Solvent B: 20 % Acetonitrile
Mode : Isocratic
Flow Rate: 1.0 ml/min
Wavelength: 265 nm

Tetracyclines at pH 2

Peak List:

1. Oxytetracycline
2. Tetracycline
3. Demeclocycline
4. Chlortetracycline
5. Doxycycline



Conditions:

Column: Pinnacle ODS Amine
Dimensions: 150 x 4.6 mm
Particle Size: 5 um
Pore Size: 120A

Solvent A: 80 % 0.05M KH2PO4 pH 2.0
Solvent B: 20 % Acetonitrile
Mode : Isocratic
Flow Rate: 1.0 ml/min
Wavelength: 265 nm

The Resolution Equation

- The ultimate goal of chromatography is to resolve two or more compounds into separate peaks.
- Resolution (R_s) is defined by the distance between two peaks relative to the widths of the peaks

$$R = \Delta t_r / W$$

The Resolution Equation

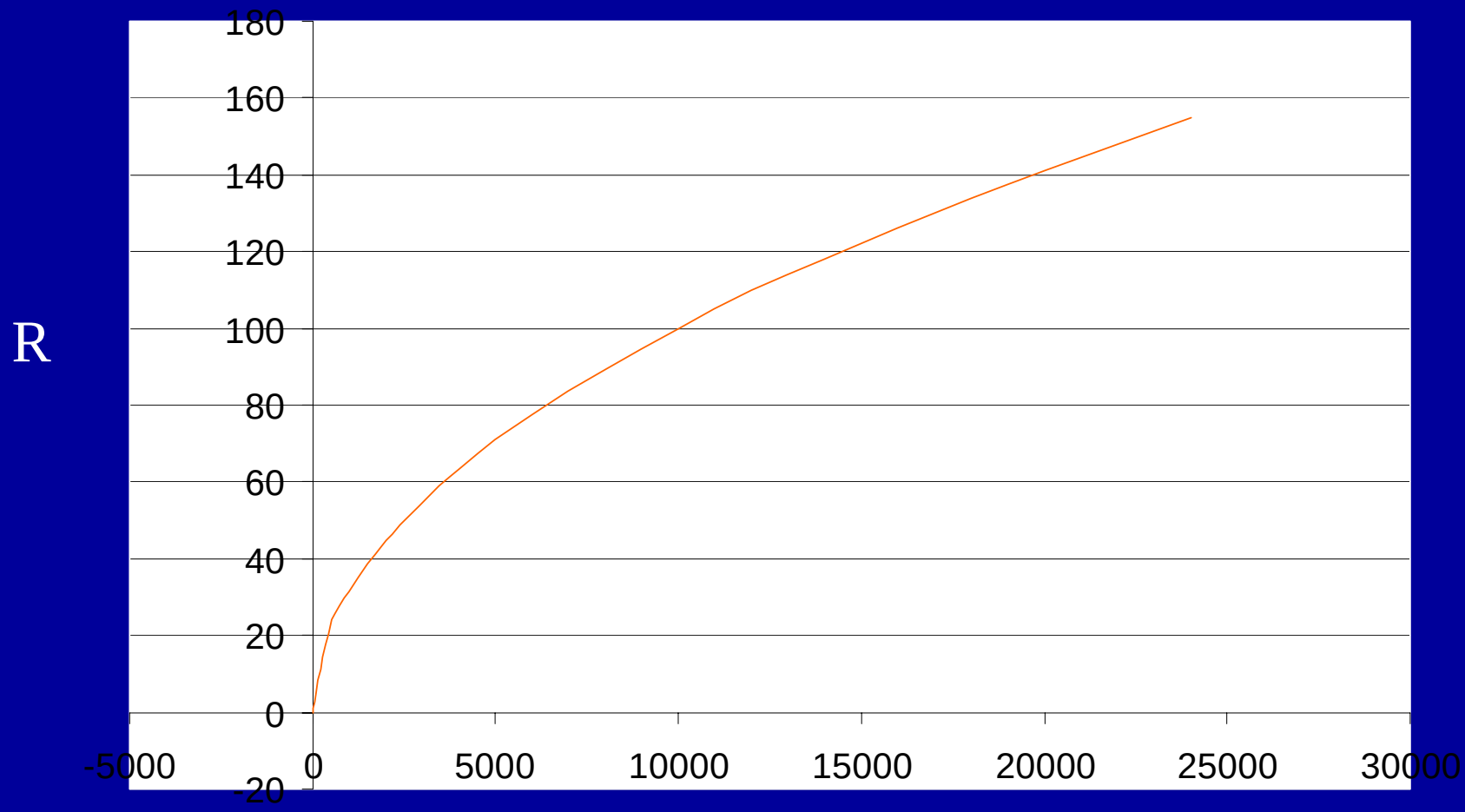
$$R = \frac{1}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\sqrt{N}\right)\left(\frac{\kappa'}{1 + \kappa'}\right)$$

Selectivity

Efficiency

Capacity

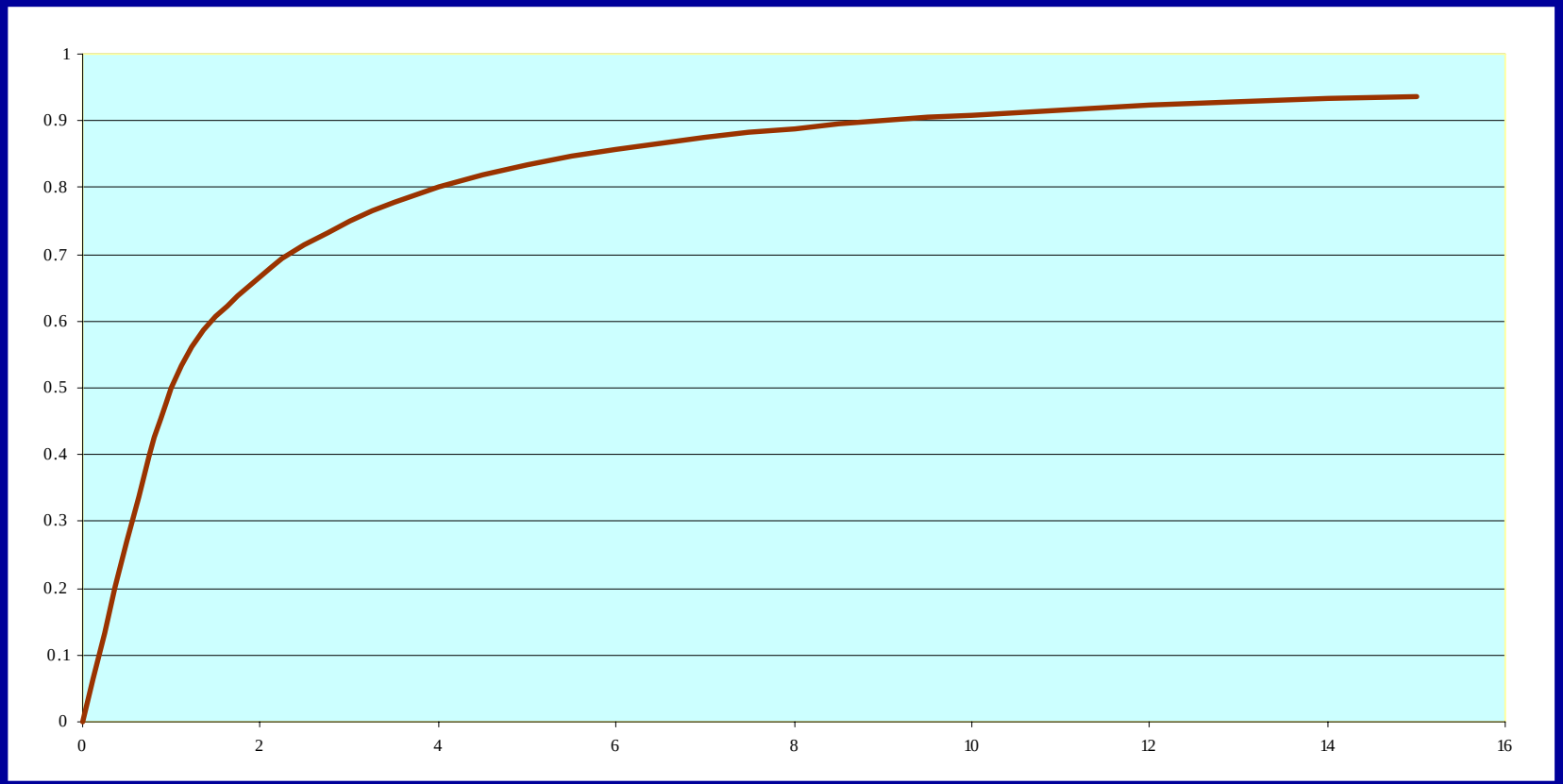
Effect of N on Resolution



Effect of N on Resolution

- Resolution is proportional to square root of N
  - To double resolution, N would have to increase by a factor of 4
- N can be increased with longer column or smaller particle size
  - $R \propto \sqrt{N}$
  - $N \propto$ column length
  - $N \propto 1/$particle diameter
  - Limited by column pressure

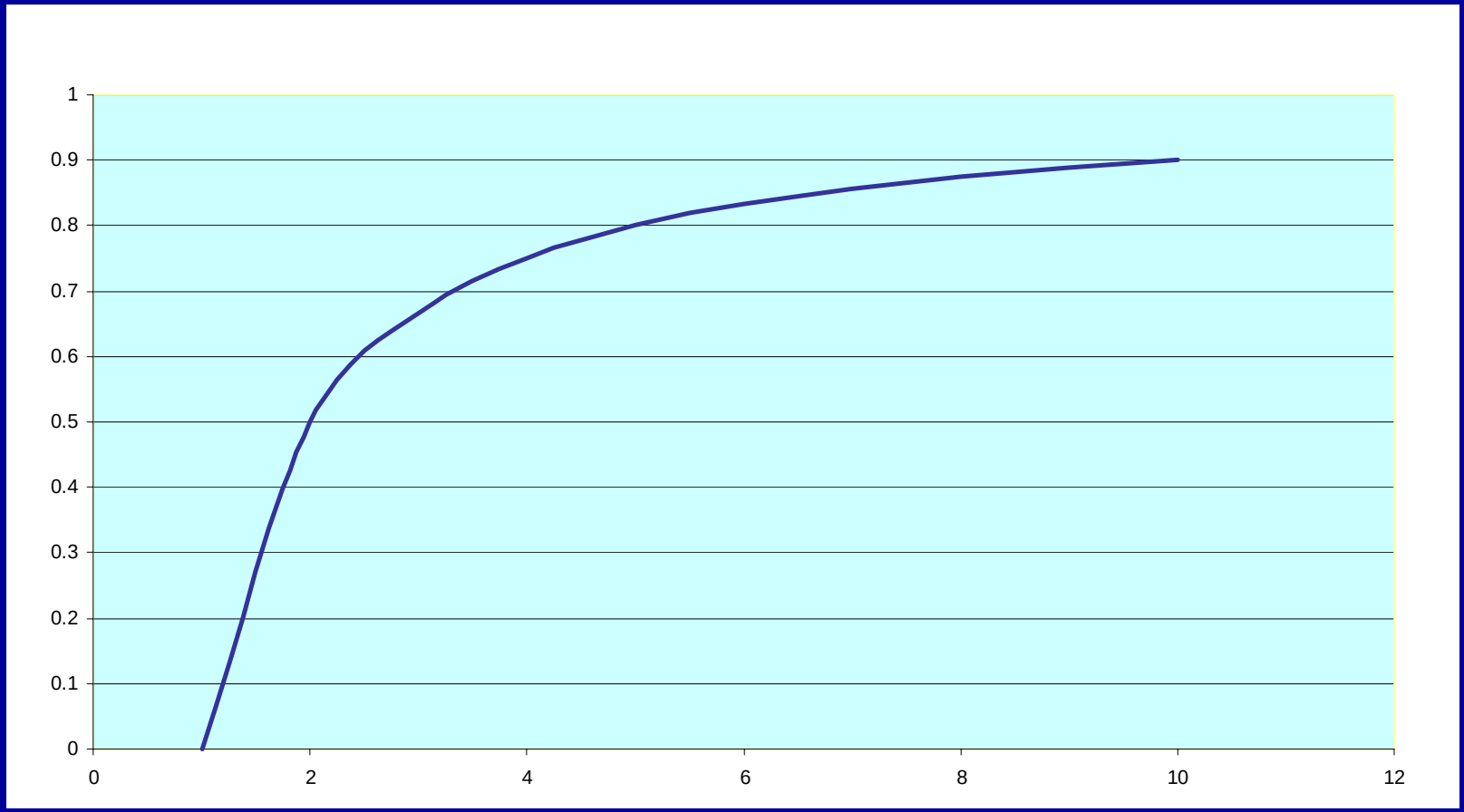
Effect of k’ on Resolution



Effect of k' on Resolution

- Practical limitation on how much R can be increased by changing k'
- Increasing k' has increasingly smaller benefit to R, especially at k' > 5
- Increasing k' comes at cost of greater analysis time
- k' is changed by altering mobile phase strength

Effect of α on Resolution



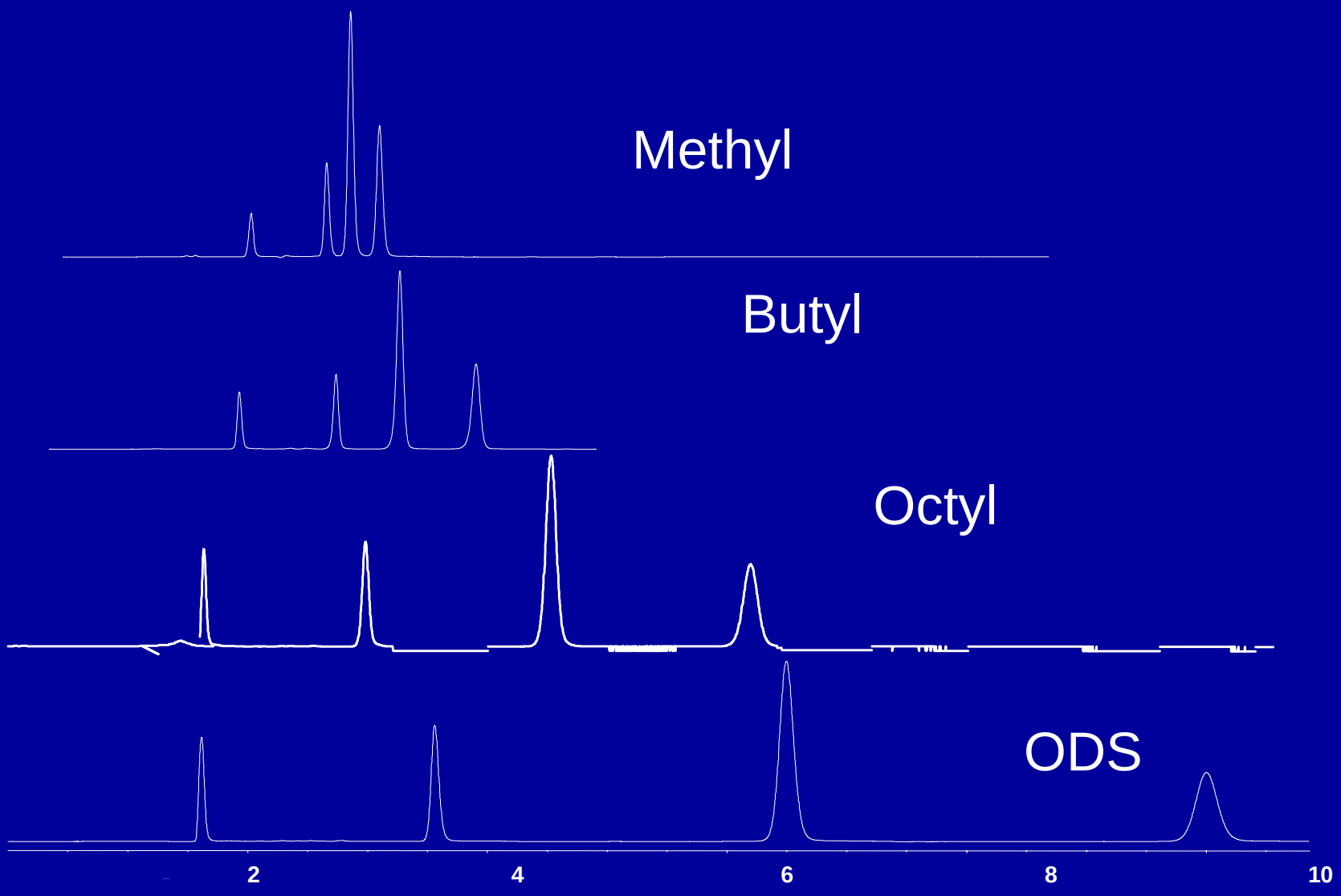
Effect of α on Resolution

- Changing α is the most effective way to increase resolution
- α can be altered over wide range without sacrificing time or higher pressure
- Adjust α by changing stationary phase or mobile phase solvents

Method Development Based on the Resolution Equation

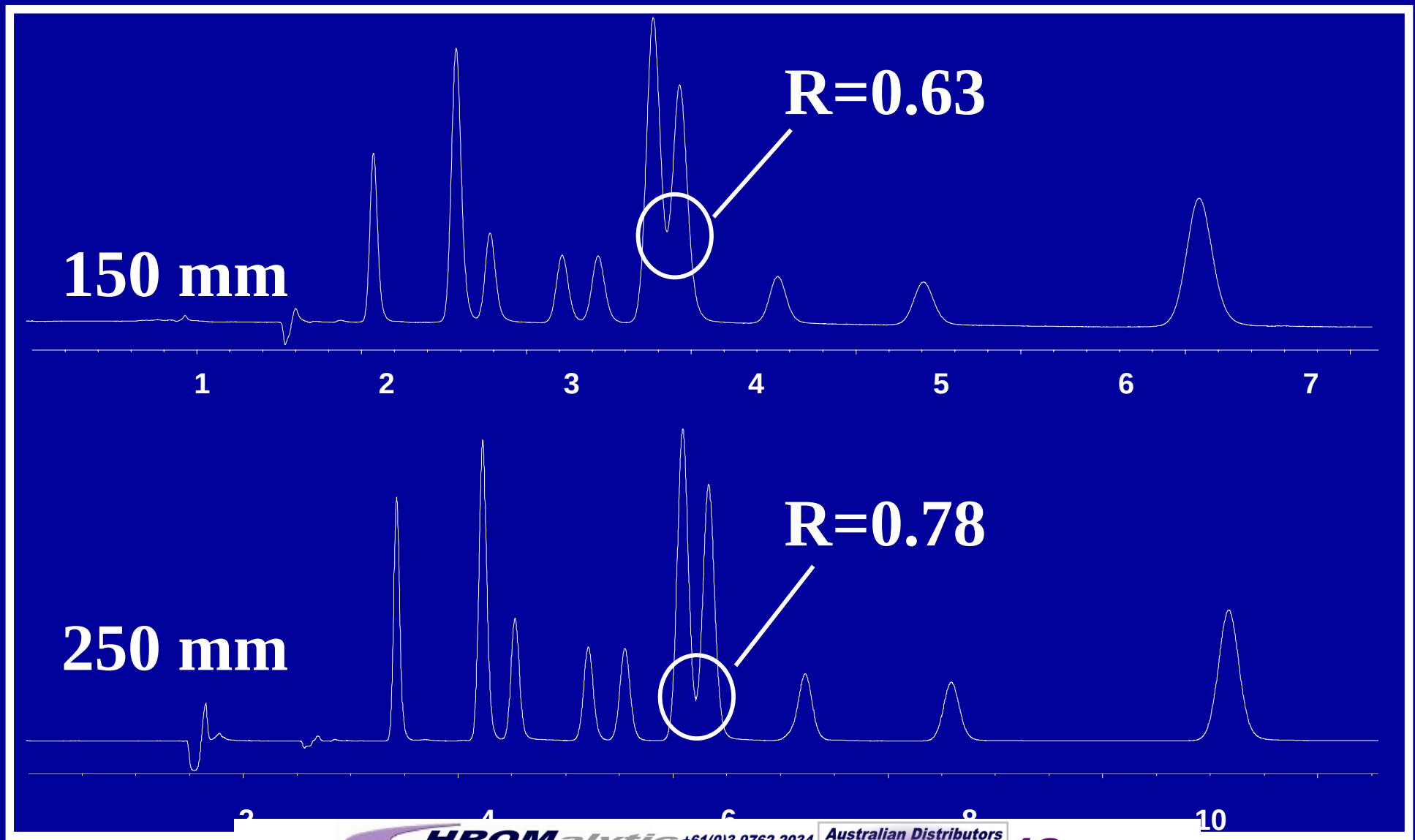
1. Adjust k' to optimum range (~2-5)
2. If not close to desired resolution, adjust selectivity by changing either mobile phase or stationary phase
   - Return to step 1
3. If close to desired resolution, increase N by increasing column length or decreasing particle size

Stationary Phase Type (α)



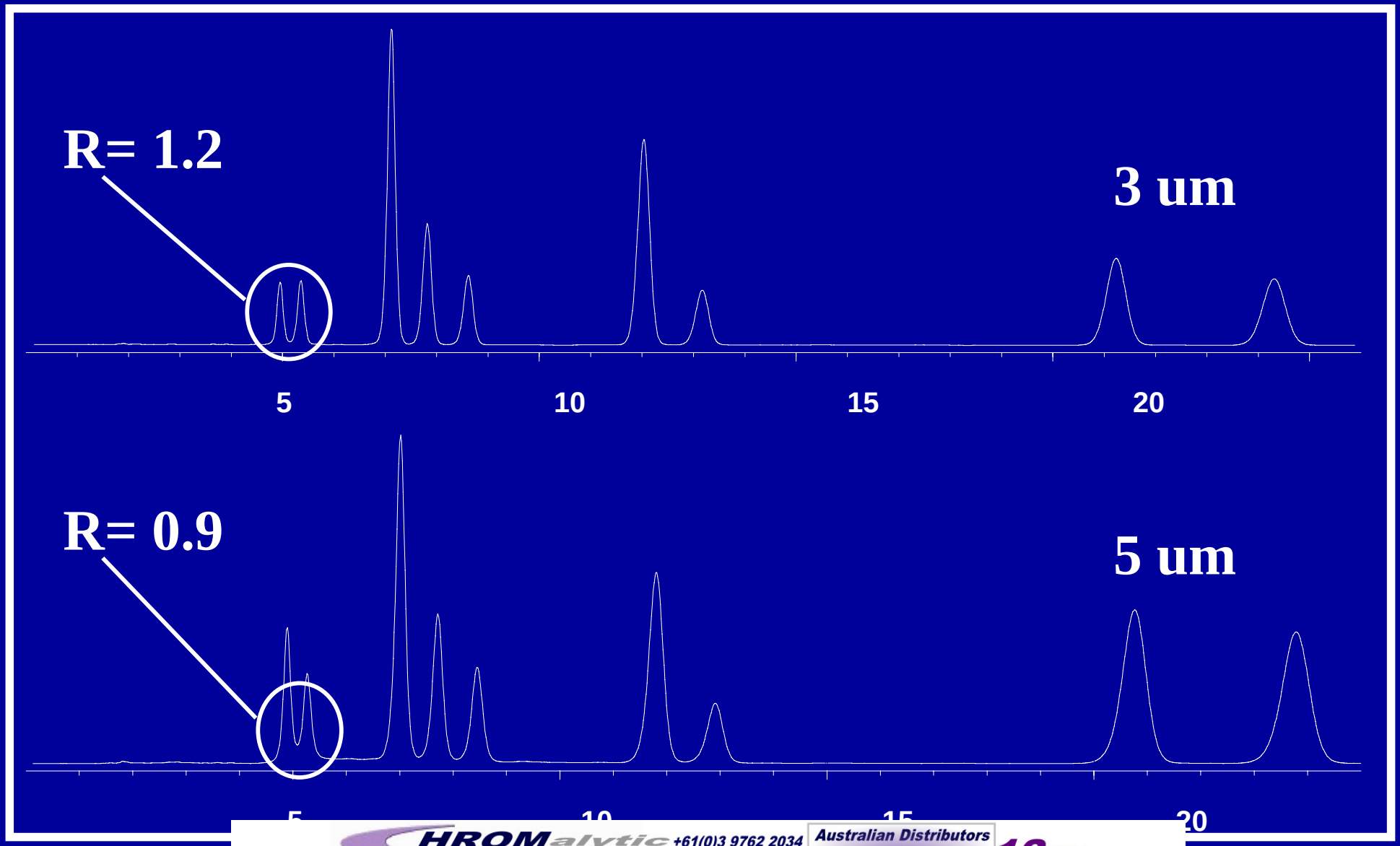
Barbiturates

150 mm vs 250 mm Length

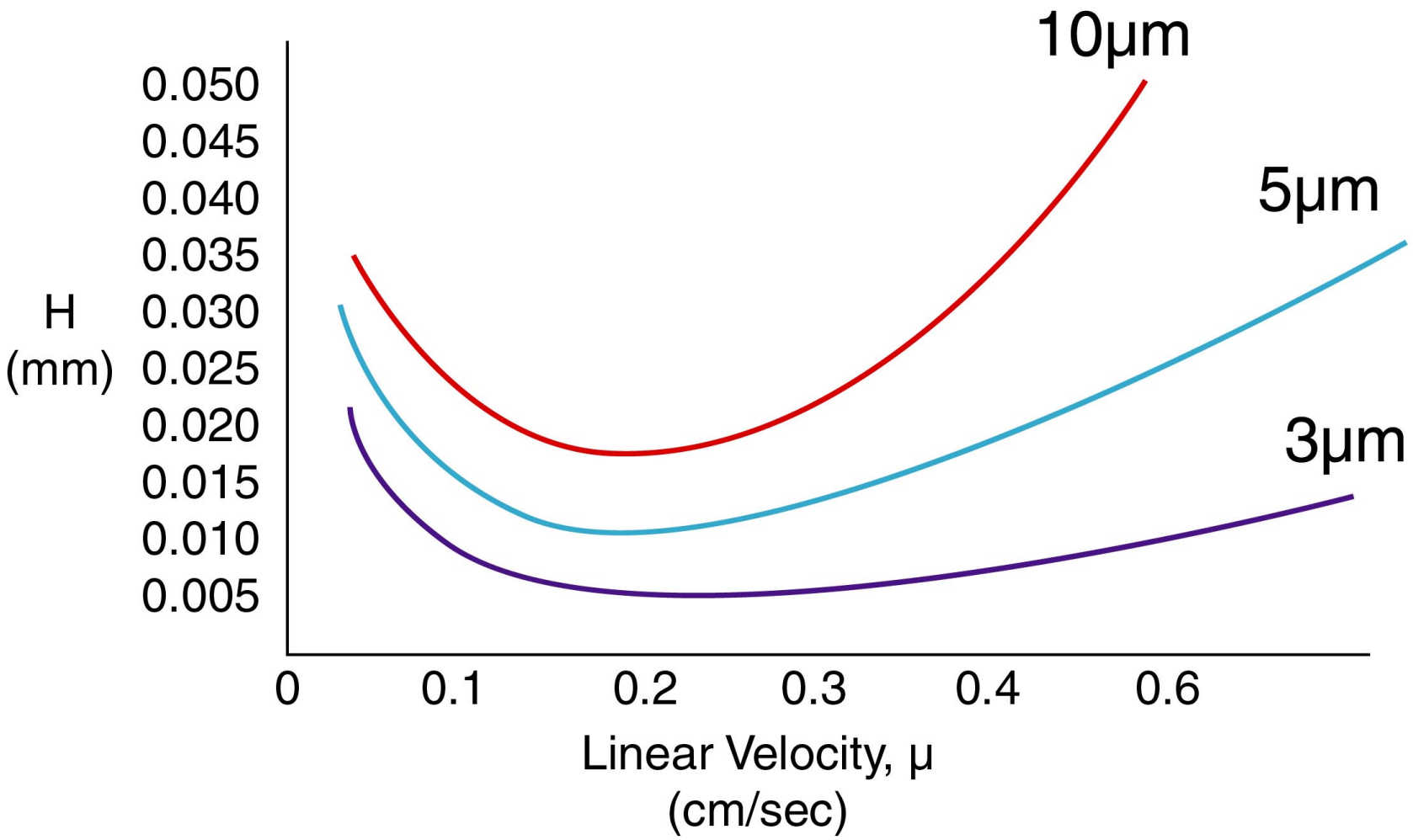


Triazines

3 um vs 5 um dp



Van Deemter Plot



Approximate Optimum Flow Rate

Column	Flow Rate (mL/min) Particle Size	
ID (mm)	3µm	5µm
1.0	0.075	0.050
2.1	0.300	0.200
3.2	0.750	0.500
4.6	1.5	1.0

Temperature Control

- Slightly above ambient temperature (i.e. 30°C) to maximize temperature stability
- Temperature affects retention and in some cases selectivity
- Increasing temperature can decrease pressure by reducing mobile phase viscosity
- Maximum recommended temperature for most columns is 80°C

HPLC Method Validation

- Ensure that the method will provide similar results over a long period of time in other labs
- Challenge the method to determine the limits of allowed variability for each method parameter
- International Council on Harmonization (ICH)
  - Guidelines for the validation of analytical methods
  - Applied universally by all agencies and all analytical methods

ICH Method Development Parameters

- Precision
- Accuracy
- Limit of Detection
- Limit of Quantitation
- Specificity
- Linearity
- Range
- Robustness
- System Suitability

Summary of Method Development

- Define goals
- Gather information
- Select mode
- Adjust k’
- Adjust α
- Optimize N if needed
- Know when to quit