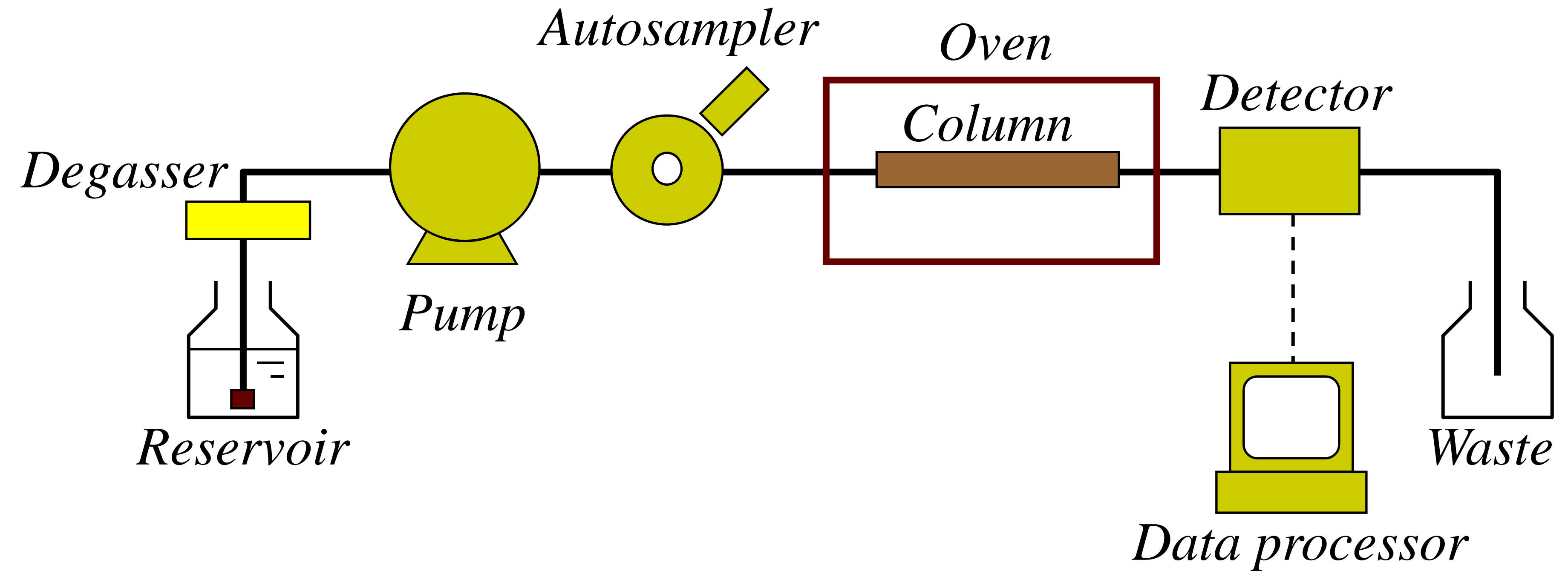
Types of HPLC

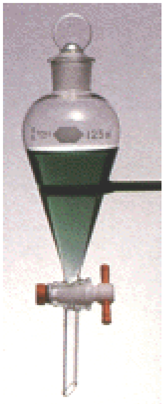
(Gradient and Isocratic)

Flow-line



Capacity factor k′

$$k' = K\frac{V_s}{V_m} = \frac{Amount}{Amount}\frac{stat.phase}{mobile}$$



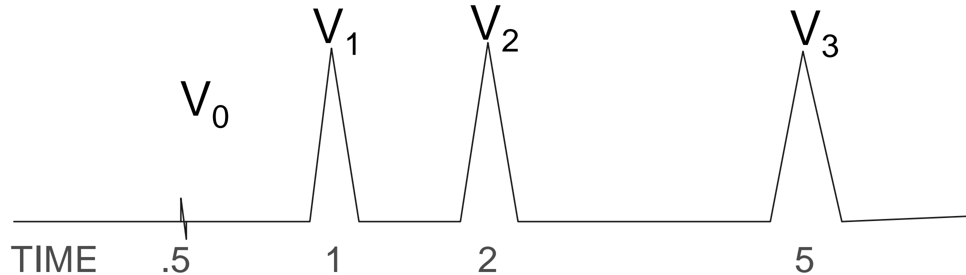
$$K = \frac{C_s}{C_m} = \frac{M_s \; V_m}{M_m \; V_s}$$

substitute

$$k' = \frac{V_r - V_0}{V_0} = \frac{t_r - t_0}{t_0}$$

k = Retention Factor

A Measure of Retention



$$k_1 = \frac{\underline{V_1} - \underline{V_0}}{V_0}$$

$$k_1 = \frac{1-0.5}{0.5} = 1$$

$$k_2 = \frac{2-0.5}{0.5} = 3$$

$$k_3 = \frac{5-0.5}{0.5} = 9$$

- Describes how far the peak is from V_0
- No dimension, independent of flow, time and column size

Isocratic and Gradient Elution

In Chromatography the samples are some times to complex , contains many compounds with a widely different polarity.

In General the compounds have following tendencies:

•Some compounds will leave column almost un retained ($K \leq 1$)

•Some compounds will leave column within a reasonable time ($1 \leq K \leq 10$)

•Some compounds are strongly retained ($K > 10$)

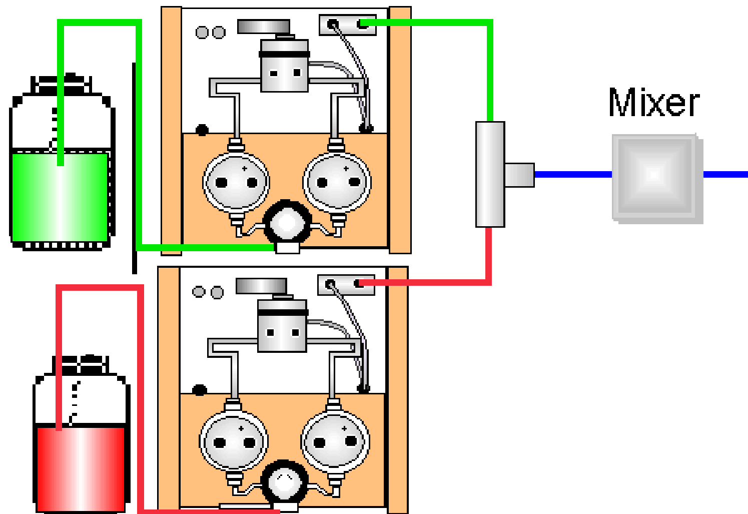
•Some Compounds are not completely separated

Gradient system

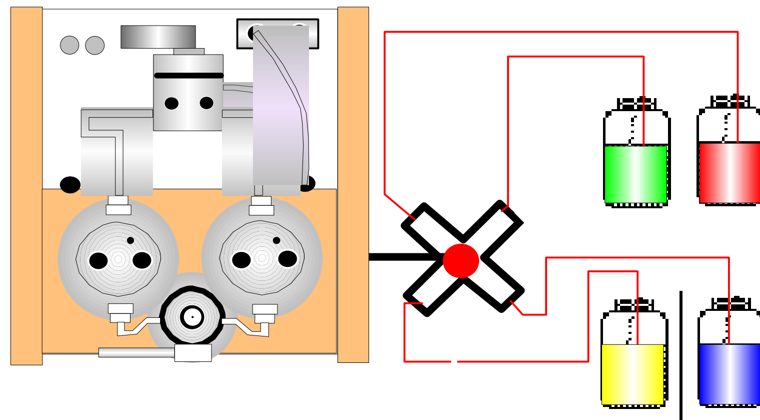
- Isocratic system
  - Fixed (un-changeable) mixing ratio during analysis
- Gradient system
  - Changeable mixing ratio during analysis
    - HPGE (High Pressure Gradient)
    - LPGE (Low Pressure Gradient)

Pumps in Gradient System

2 pump gradient system
Mixing on high pressure side



1 pump gradient system
Mixing on low pressure side



Gradient proportioning valve

Gradient Systems

“High-pressure mixing”

- Advantages
  - Usually lower system volume
  - Degassing not as critical
- Disadvantages
  - One pump per solvent
  - Only practical with up to 3 solvents (usually only 2)

“Low-pressure mixing”

- Advantages
  - Only one pump
  - Usually more solvents (normally 4)
- Disadvantages
  - Usually higher system volume
  - Degassing more critical

Aim of gradient
- problems in isocratic mode -

- in isocratic mode

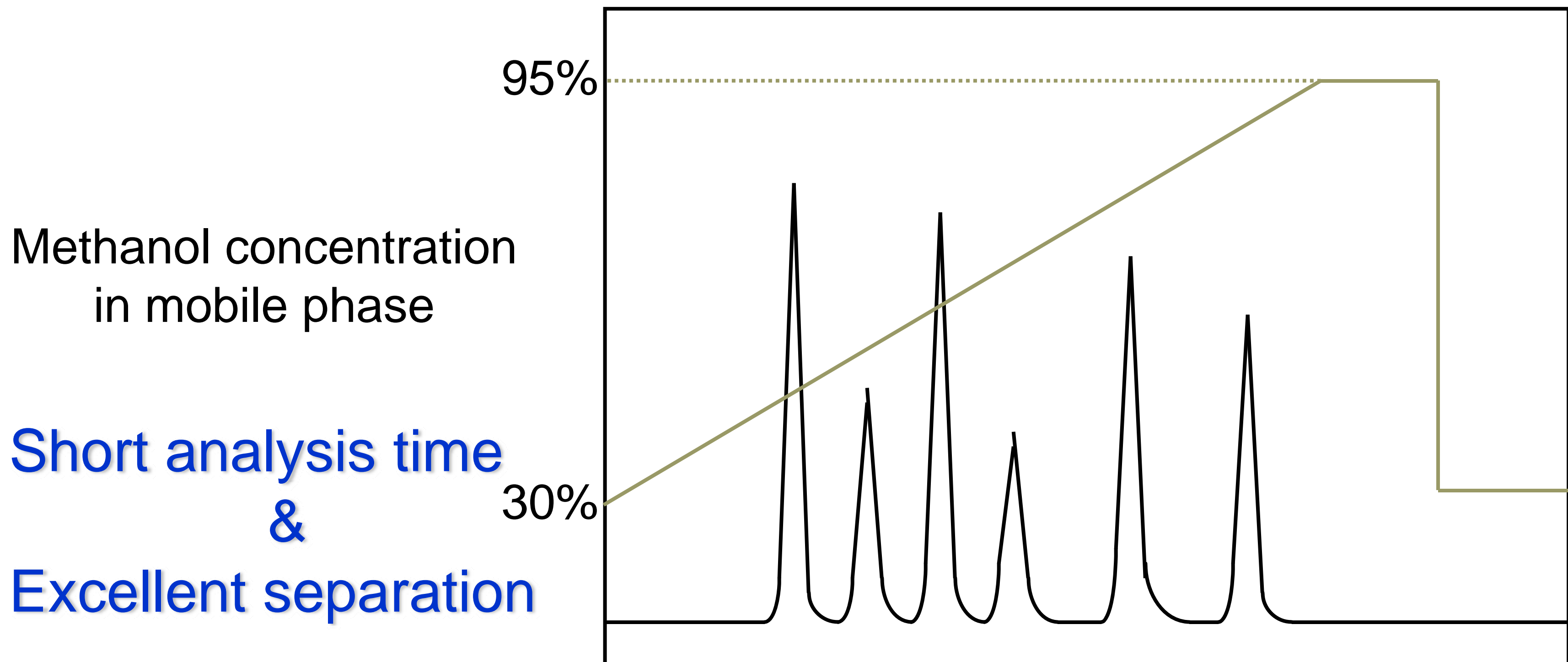
Methanol / water = 6 / 4

Methanol / water = 8 / 2

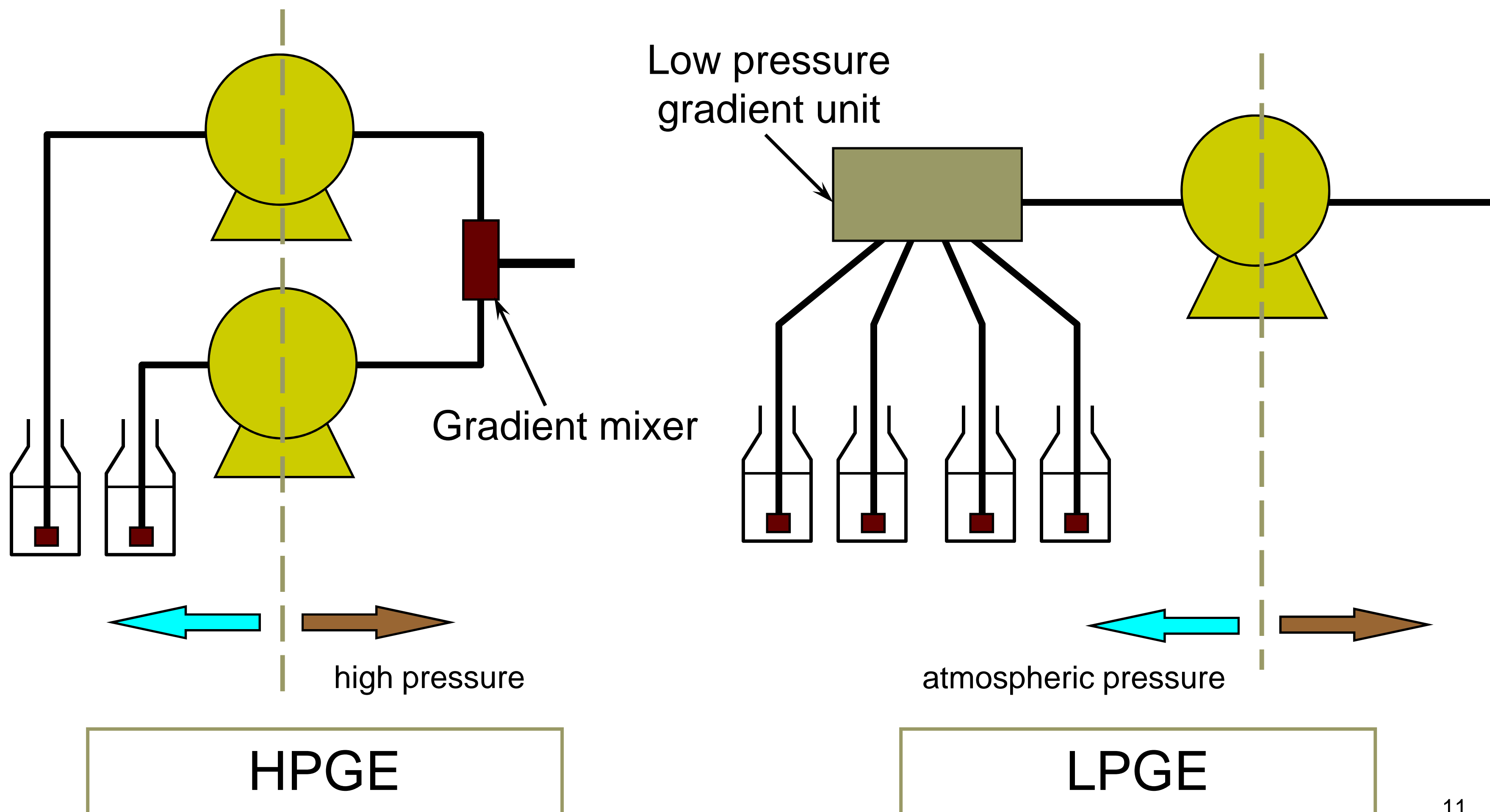
(Column : ODS type)

Aim of gradient - solution -

- Gradual change of the mixing ratio during analysis

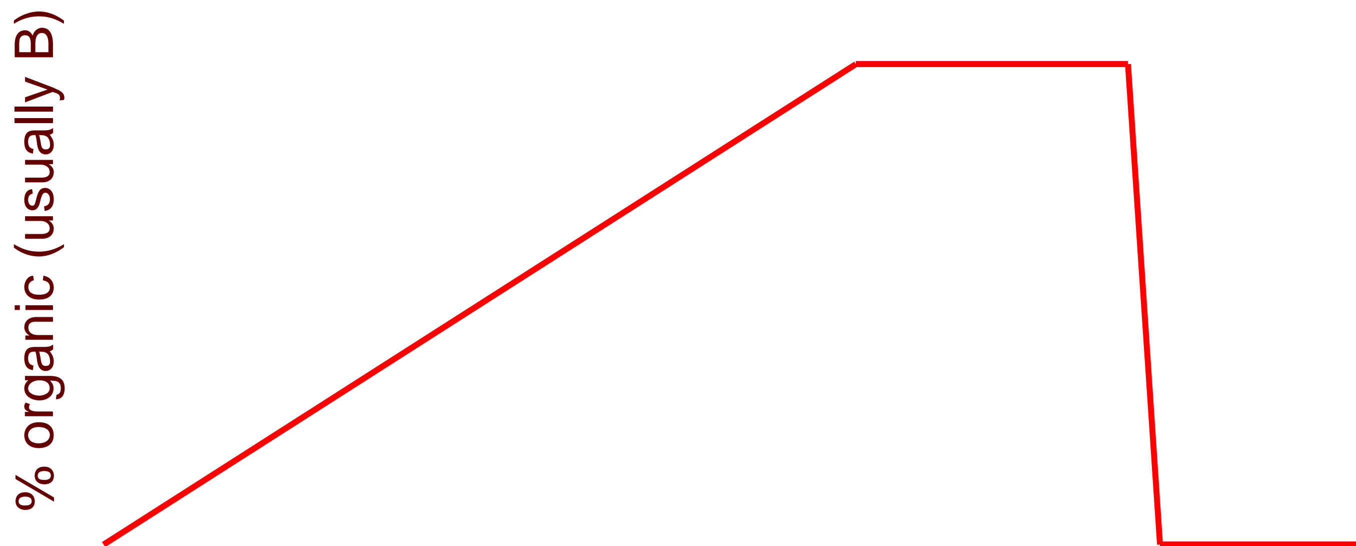


High / Low pressure gradient system



What is gradient elution ?

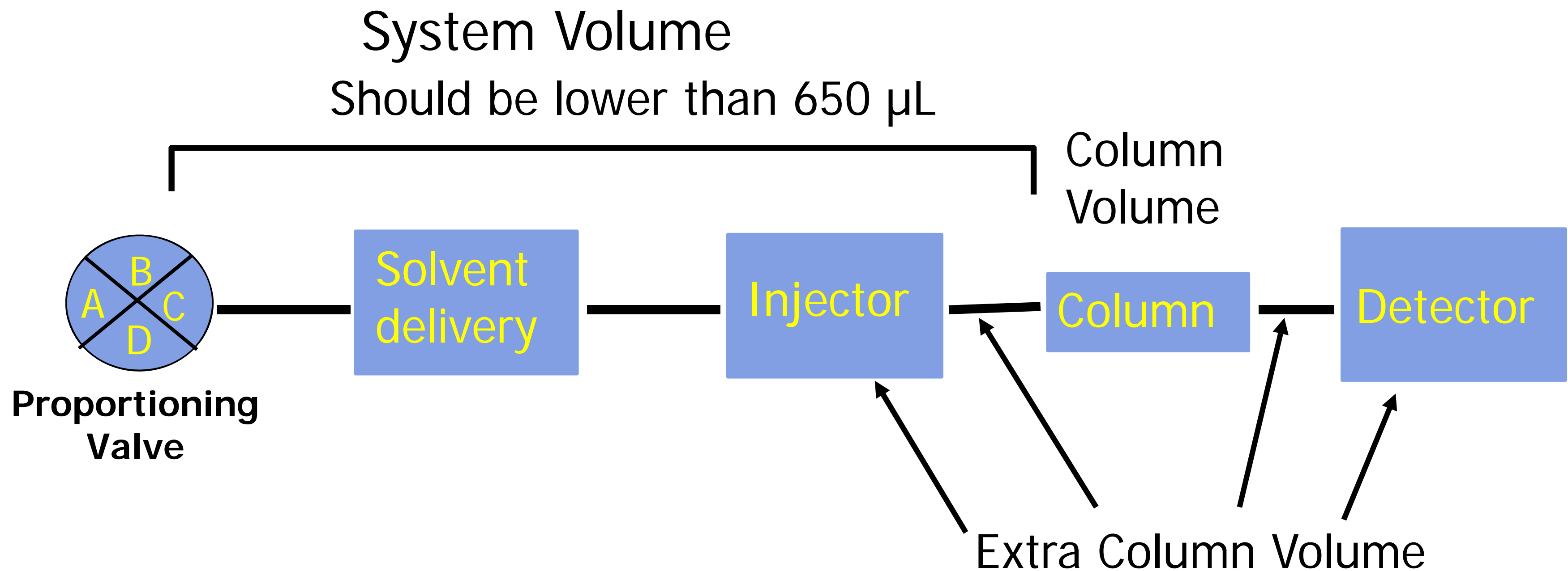
- Changing the eluting strength of the mobile phase during the run



Gradient Elution

- Separates samples with a wide polarity range.
- Gives chromatograms with sharp peaks throughout.
- Is often required for proteins and peptides.
- Gives “generic” separations for LC-MS and LC-MS-MS.
- Provides convenient procedure to develop isocratic separation conditions.

Volumes in an HPLC System



Gradient Table - Simple

TIME	FLOW	% A	% B	%C	% D	CURVE
INITIAL	1.0	80	20	--	--	*
20	1.0	50	50	--	--	6

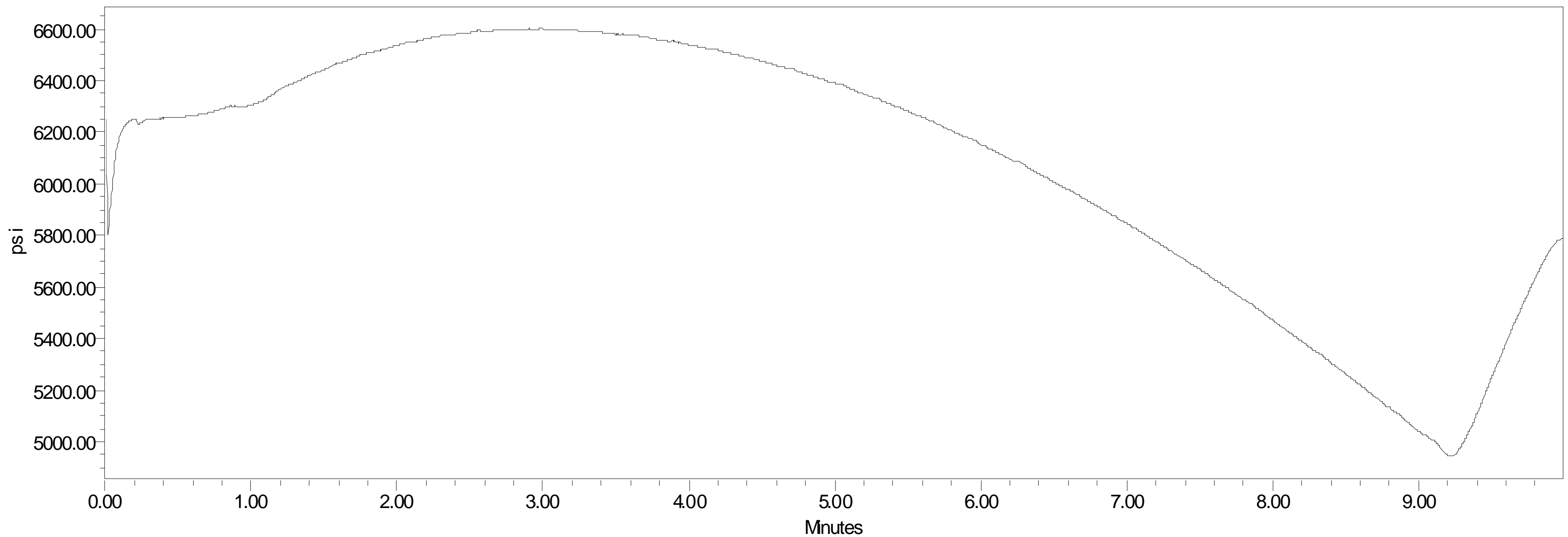
Gradient Table with Column Wash

TIME	FLOW	% A	% B	%C	% D	CURVE
INITIAL	1.0	80	20	--	--	*
20	1.0	50	50	--	--	6
21	1.0	0	100	--	--	6

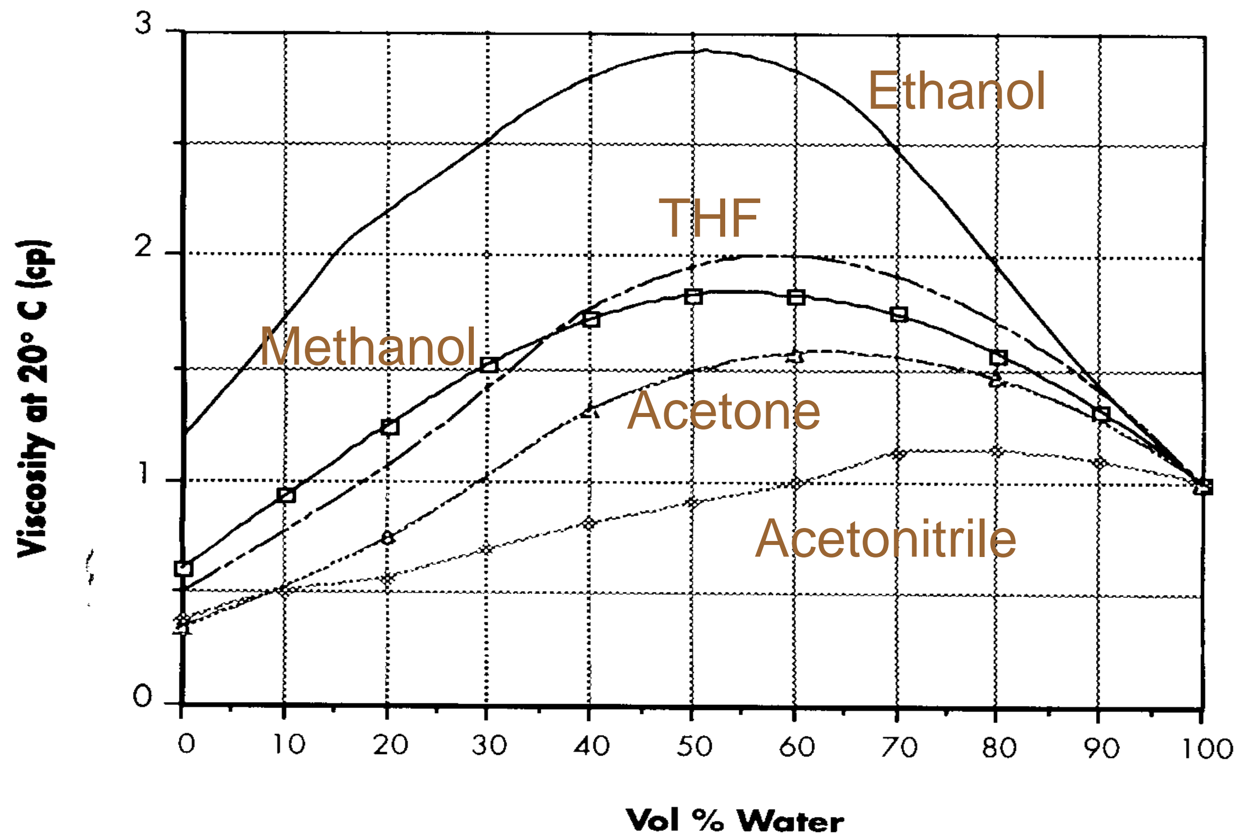
Gradient Table - Return to Initial

TIME	FLOW	% A	% B	%C	% D	CURVE
INITIAL	1.0	80	20	--	--	*
20	1.0	50	50	--	--	6
21	1.0	0	100	--	--	6
26	1.0	80	20	--	--	11

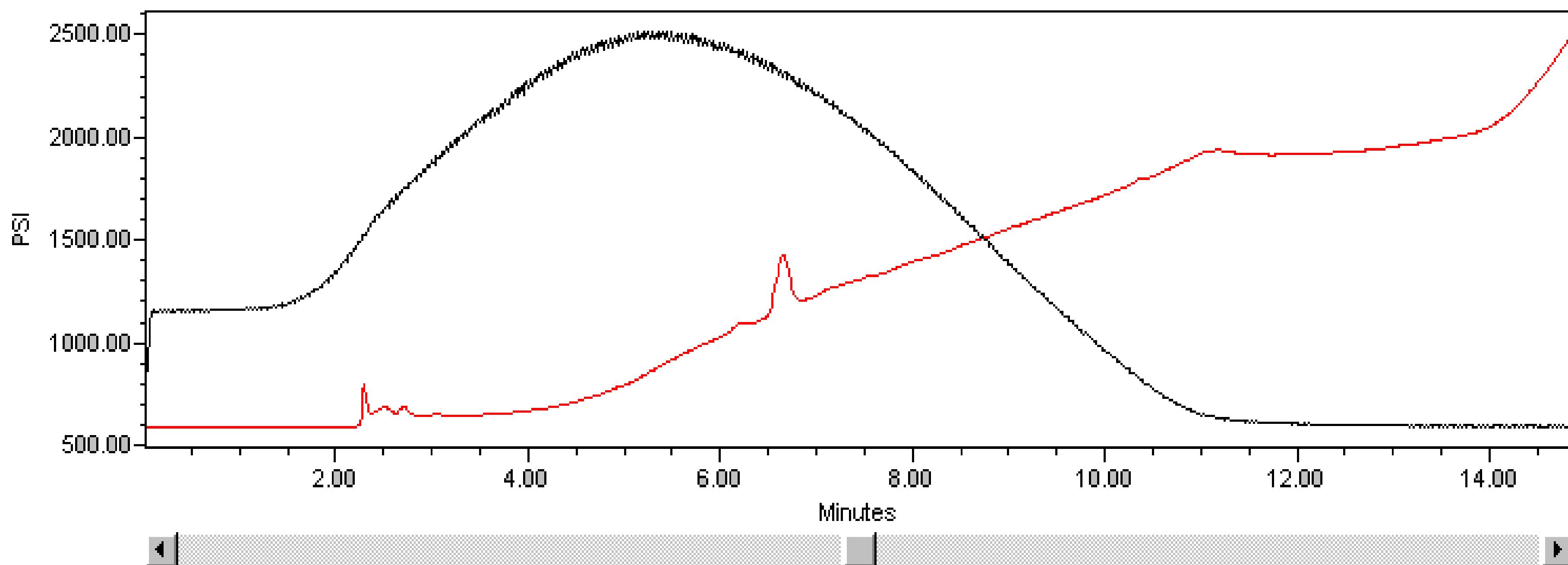
Pressure Curve 0 to 100 AcN



Viscosity for mixtures of water and organic solvents



Pressure/ Ghost Peaks



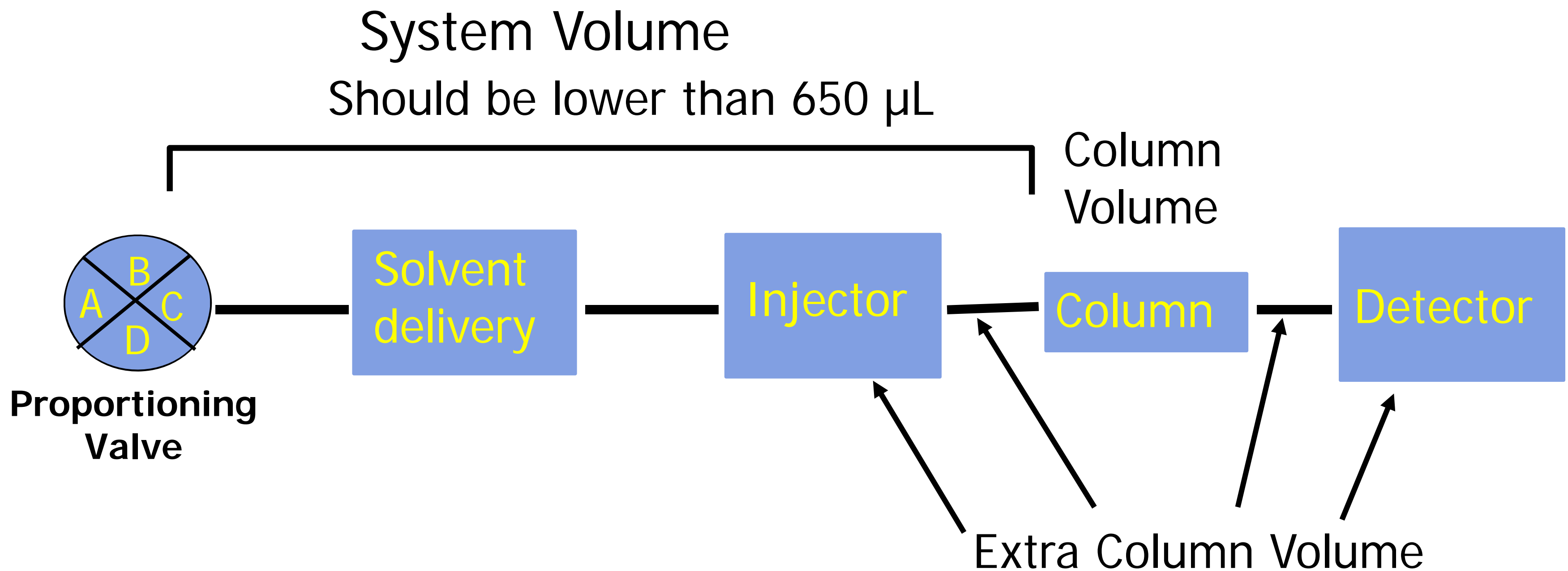
0 to 100 % THF

Curve 6

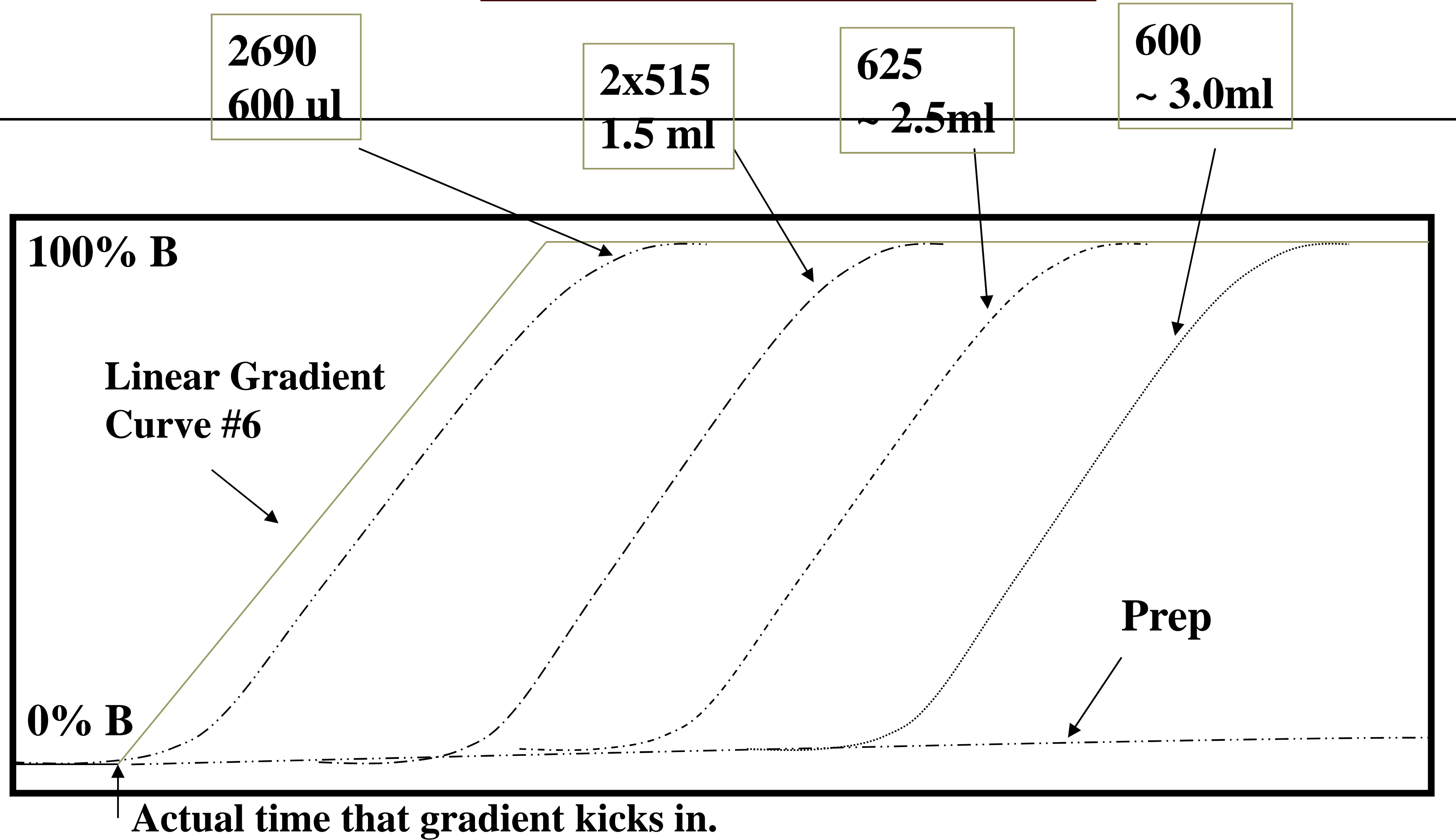
1 ml/min.

10 min.

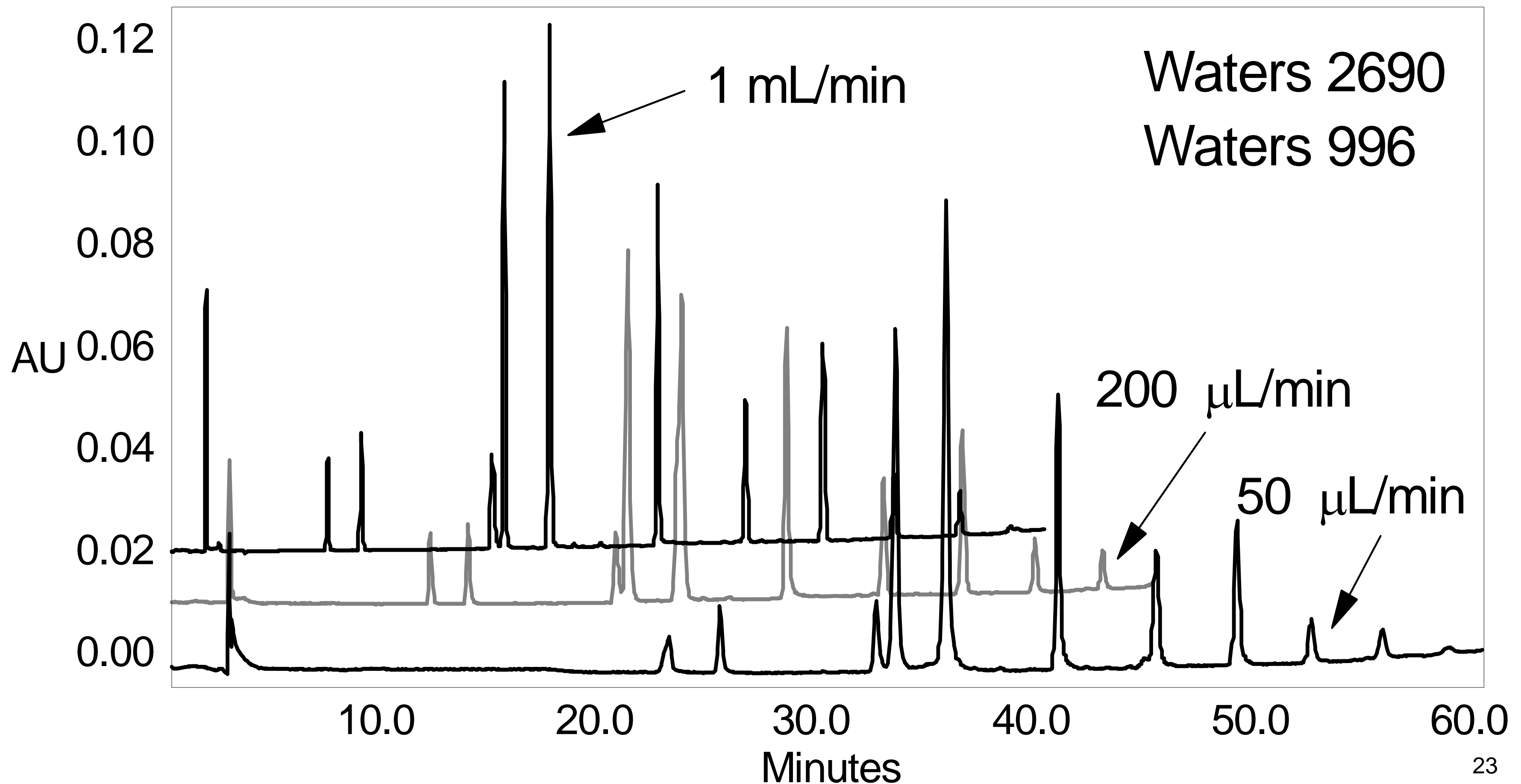
Volumes in an HPLC System



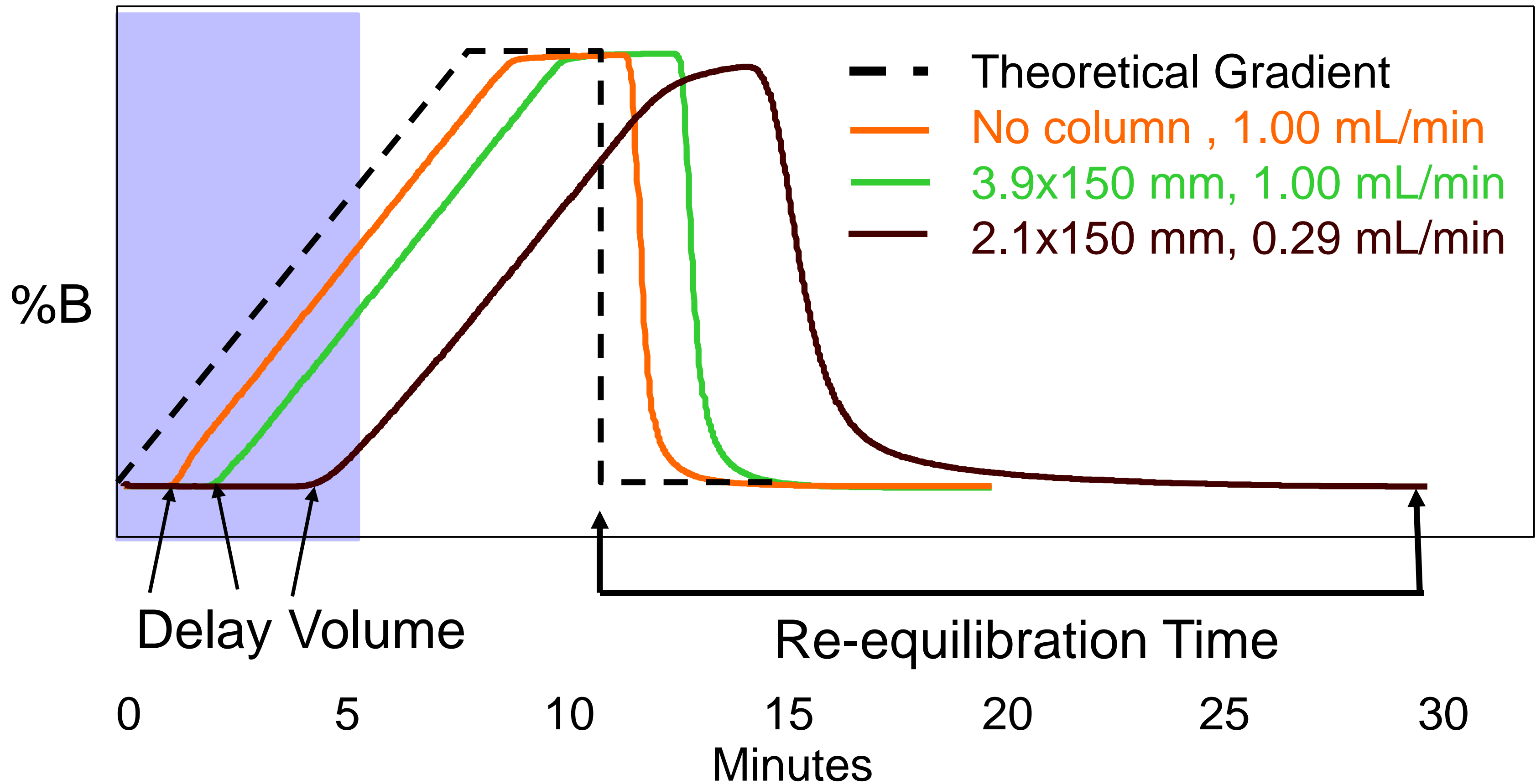
Delay Volume



Effect of Gradient Delay Volume on Peak Elution

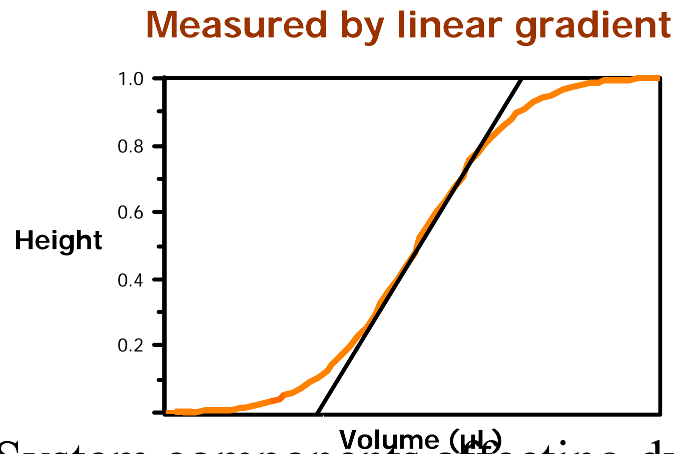


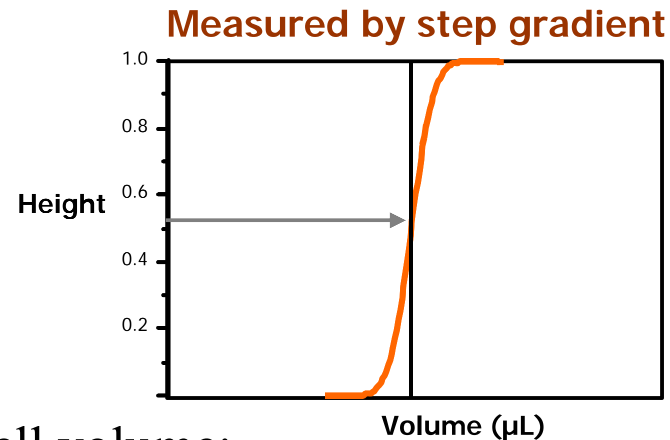
Gradient Shape and Pre-column Volume



Determination of System Pre-column Volume

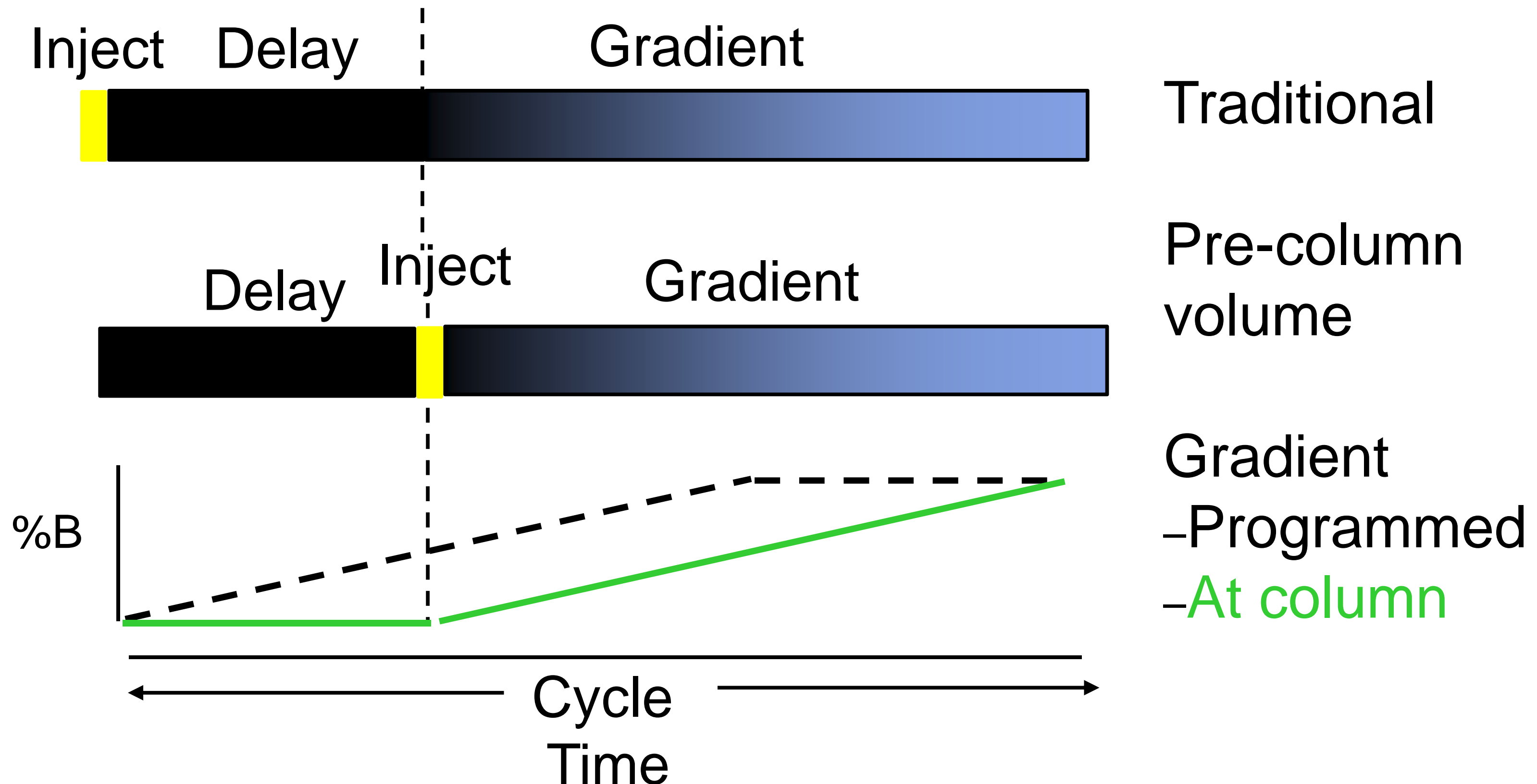
- Definition: Delay volume is the volume of plumbing between the point the gradient is formed and the inlet of the column.

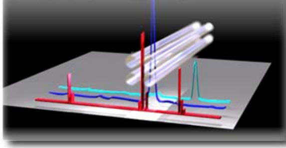




- System components affecting dwell volume:
  - Pump
  - Gradient Mixers
  - Injector

Pre-column Volume Programming Narrow bore Chromatography

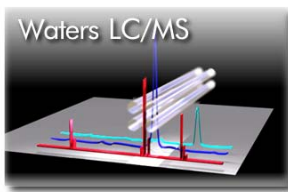




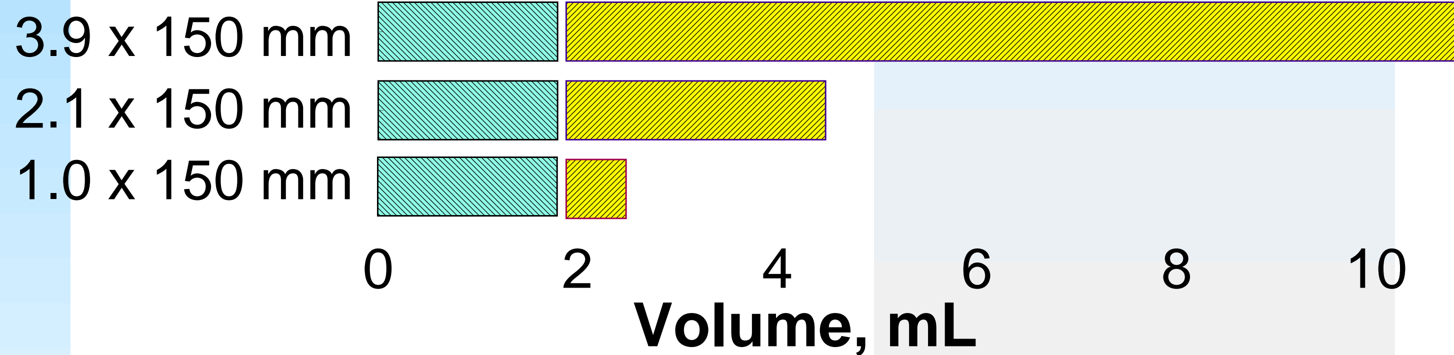
Calculation of Gradient Equilibration Volume

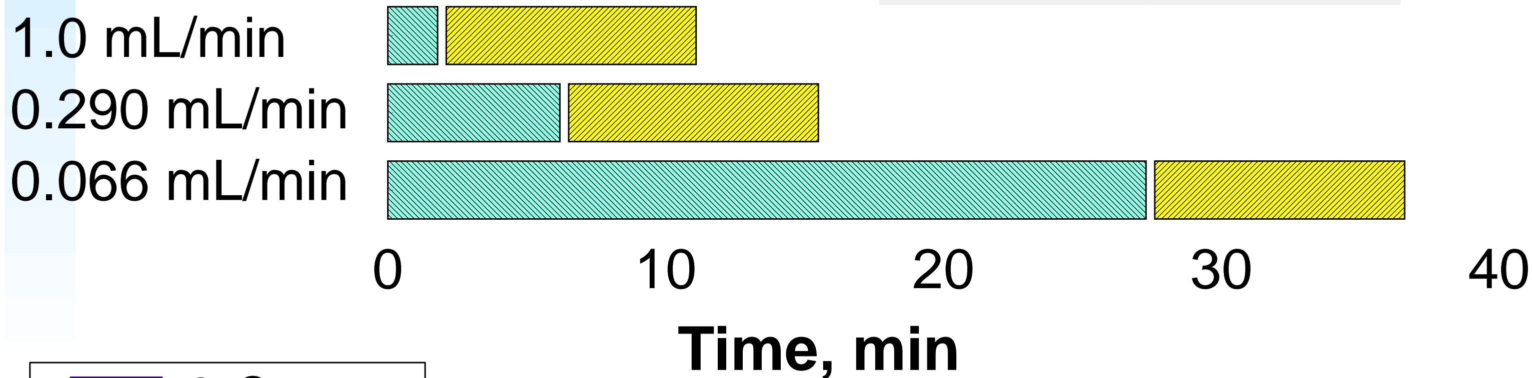
2690 System volume: measured (~600 uL)
Column volume = π R^2 * L

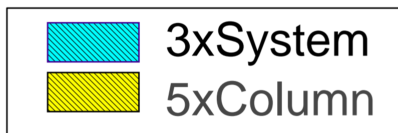
For good column equilibration
(3 x System volume) + (5 x Column volume)

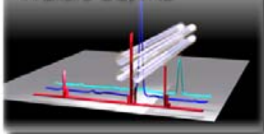


Column Re-equilibration









Column Parameters

Column Diameter mm	Column Length mm	Column Volume mL	Flow Rate mL/min
3.9	150	1.89	1.000
2.1	150	0.52	0.290
1.0	150	0.12	0.066

Calculation of Gradient Equilibrium volume

Low Pressure Gradient System (Alliance 2695)				
System Volume (ml)	0.65			
Column Dia. (mm)	Column Length (mm)	Column Volume (ml)	Good Column Equilibrium=(3X system Vol.)+(5xColumn Vol.)	Flow Rate (ml)
3.9	150	1.79	11	1.0
3.9	300	3.59	20	1.0
4.6	250	4.16	23	1.0

High Pressure Gradient System (Breeze 1525 Gradient)				
System Volume (ml)	0.20			
Column Dia. (mm)	Column Length (mm)	Column Volume (ml)	Good Column Equilibrium=(3X system Vol.)+(5xColumn Vol.)	Flow Rate (ml)
3.9	150	1.79	10	1.5
3.9	300	3.59	19	1.5
4.6	250	4.16	21	1.5

Calculation of Gradient Equilibrium volume

UPLC				
System Vol. (ml)	0.16			
Column Dia. (mm)	Column Length (mm)	Colume Volume (ml)	Good Column Equilibrium=(3X system Vol.)+(5xColumn Vol.)	Flow Rate (ml)
2.1	50	0.17	1	0.6
2.1	100	0.35	2	0.6

Gradient Elution in HPLC

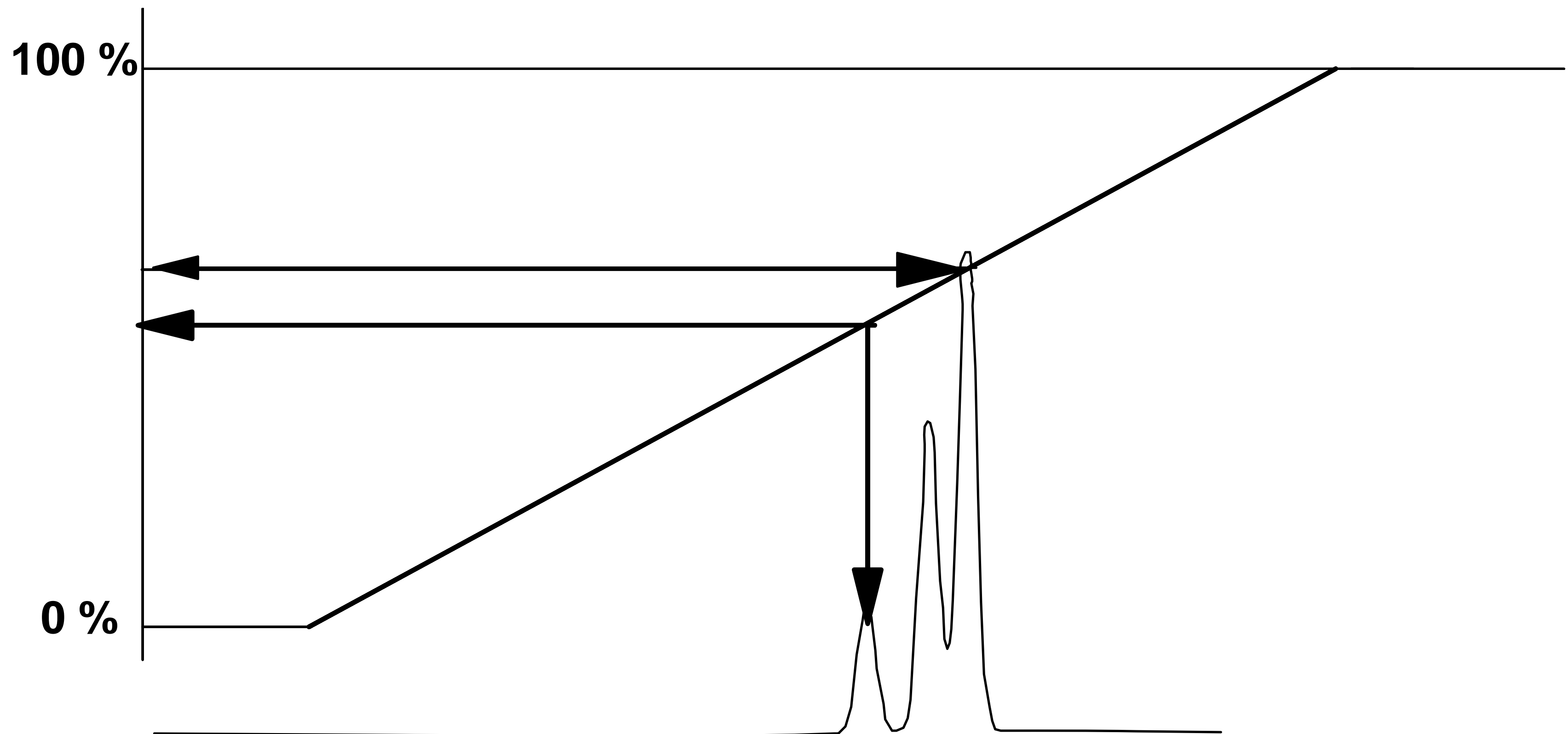
- Advantages
  - Shorter analysis Time
  - Optimal separation of all compounds
  - Improved sensitivity
  - Suitable for Complex mixtures
  - Cleaning of the Column after each run
  - For method development

Gradient Elution in HPLC

- Disadvantages
  - Stabilization time after each run
  - Reduced column Life Time
  - Baseline Problems
  - More critical degassing of solvents
  - Not for all detectors, like RI
  - Mixing Problems in Pump

- Use a 2% per minute gradient.
- Determine the instrument delay time.
- Calculate the mobile phase that elutes the first peak from the column.
- Calculate the mobile phase that elutes the last peak from the column.
- Take the average value.

Gradient Method Development



Calculation Continued

- Assume a one minute delay.
- Add 20% initial conditions
- Peak 1: 19-1 min. at 2%/min. = 36+20 = 56%
- Peak 4: 33-1 min. At 2%/min. = 64+20 = 84%
- (56% + 84%)/2 = 70%

Manual Calculation of Isocratic Mobile Phase

- Estimate the gradient strength when the first and last peaks eluted.
- Include a delay time for the system and column.
- Calculate the average mobile phase concentration.

Calculate Isocratic mobile Phase

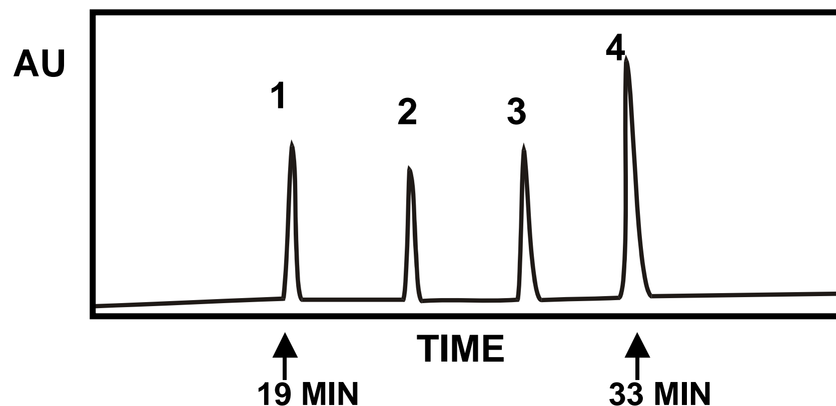
- From gradient six - 2% per min. :
- 2.4 min.-1 min. x 2% + 60% = 62.8% THF
- 10 min.- 1 min. x 2% + 60% = 78% THF
- Best isocratic mobile phase = 70.4% THF

Gradient Runs at ph 7.0

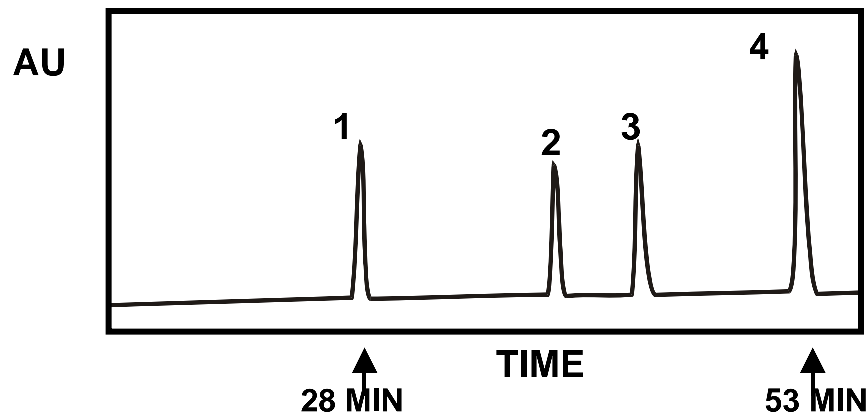
Column:	Symmetry C18 3.9 mm x 150 mm
Temp:	35º C
Gradient:	from 20% MeOH/80% 20 mM potassium phosphate buffer to 80% MeOH/20% buffer
Flow rate:	1 ml/min
Detection:	UV at 254 nm

1. Nordoxapin
2. Doxapin
3. Nortriptyline
4. Amitriptyline

GRADIENT SLOPE: 2%/min



GRADIENT SLOPE: 1%/min



‘Ghost’-Peaks with gradient elution

Gradient Blank:

Unless we are certain, a gradient should always be carried out first without injection of a sample to verify if the eluent used are sufficiently pure.

In General, solvents that are not HPLC-grade can contain organic impurities which concentrate in the first part of the gradient (if mobile phase contain water), at the top of column packing. As the amount of organic modifiers increases, these compounds will finally elute. That is why the baseline disrupted by ghost peaks which can have a negative effect on separation of the peak of interest.