Fast AND Sensitive: Automating High Throughput Analysis with Capillary HPLC

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Why Use Capillary Liquid Chromatography?

- Capillary LC systems have been developed to improve the sensitivity of separations by
  - Increasing the concentration of the analyte in the peak
    - Sample is eluted in a smaller amount of solvent
    - Improves sensitivity in concentration-dependent detectors such as mass spectrometers
  - Providing increased electrospray ionization efficiency in the mass spectrometer interface
    - Low flow rates allow for smaller droplets to form in ESI thus improving efficiency
    - Sample stream can be directly coupled to the MS interface

Trends in Capillary LC

- Capillary LC is popular with protein chemists for peptide analysis
  - Used for analysis of peptide digests to identify protein spots isolated by 2D gel electrophoresis
  - Sensitivity must be maximized to detect sub-picomole amounts of sample
  - Sample must be conserved due to its limited supply
- Little emphasis given to capillary LC in bioanalysis
  - Concern given to turnaround time and the number of samples able to be performed per day

Capillary LC in Bioanalysis

- However…as pharmaceutical development is increasingly directed at higher potency drugs, analysis sensitivity is becoming more important

- To gain this sensitivity, the chromatographic system can be scaled down

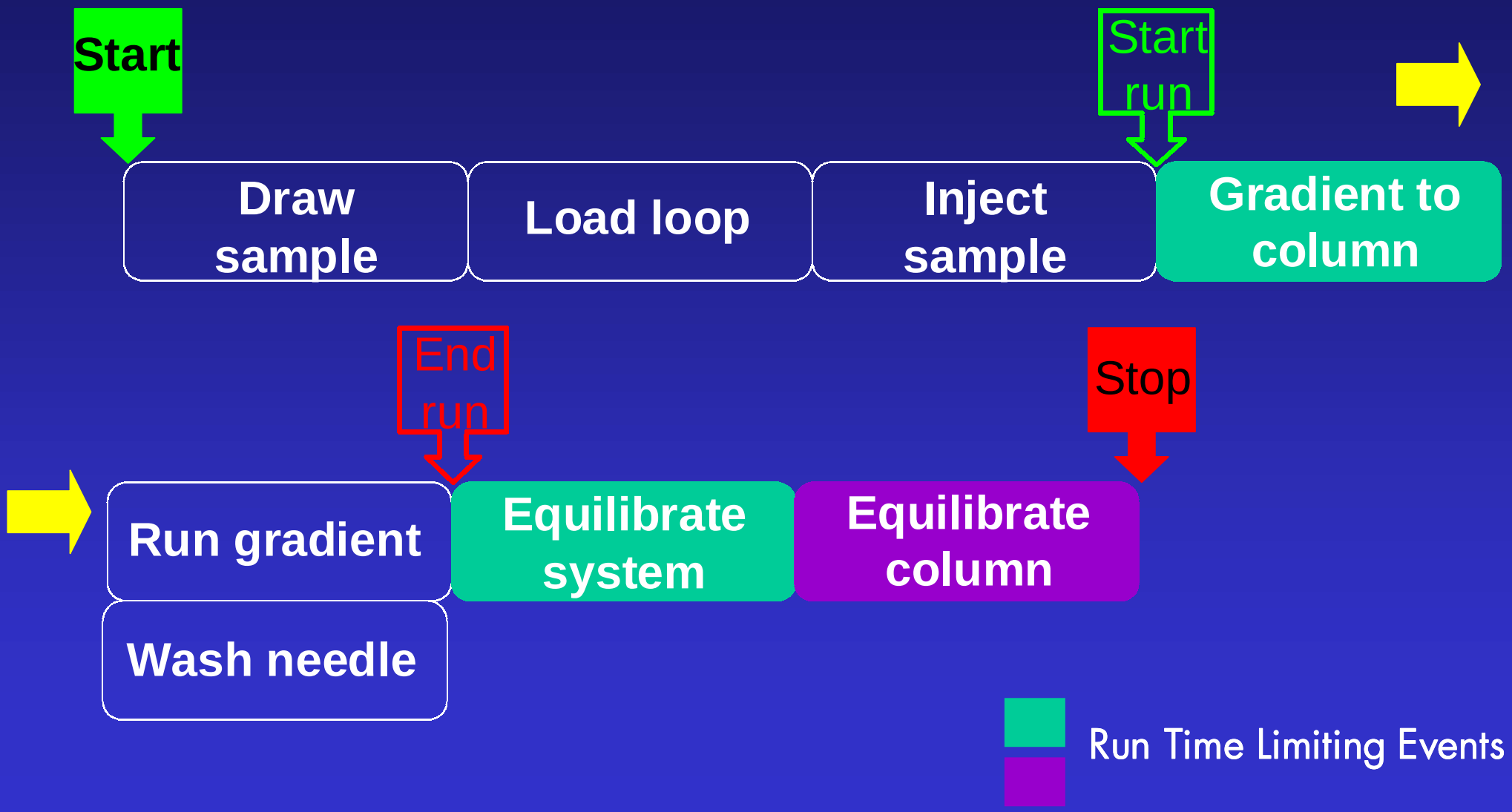
Capillary LC

Scaling of Chromatographic Systems

By decreasing the internal diameter of the column, sensitivity increases substantially

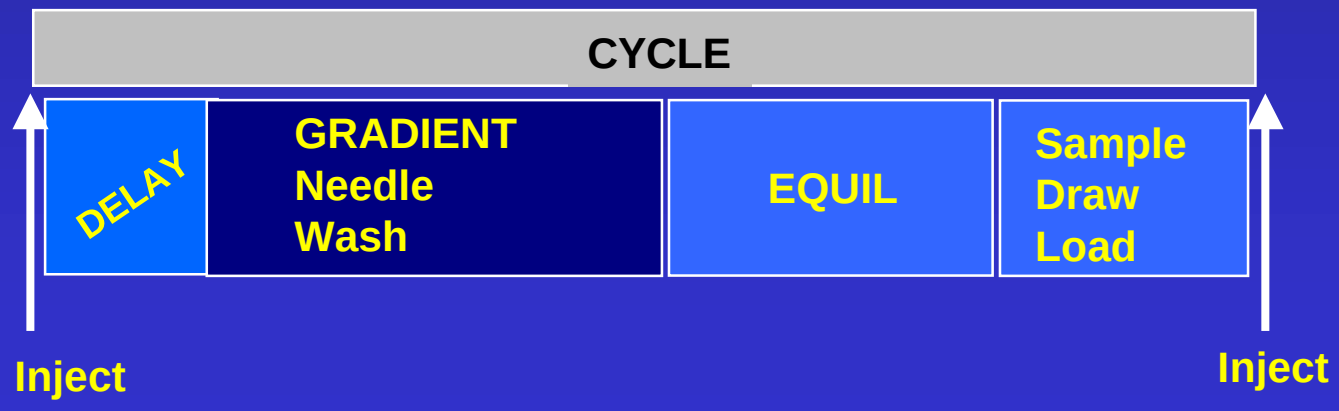
Column Dimensions	Theoretical Factor of Improvement in Sensitivity	
4.6 x 50 mm	1	1
2.1 x 50 mm	4.8	
1.0 x 50 mm	4.4	
0.32 x 50 mm	9.8	207

Events in a Chromatographic Run



Considerations with High Throughput Analyses

- Autosampler needle wash cycle
- Effects of system volume
  - Gradient delay during analysis
    - At a flow rate of 30 µl/min, 30 µl of dead volume before column will yield a 1 minute delay in the gradient reaching the column
      - Take into consideration the tubing, mixers, and loop size
  - Re-equilibration time



Re-Equilibration Time

4 µl Column Volume
+
30 µl System Volume

5x Equilibration

170 µl

At 30 µl/min, ~6 minutes re-equilibration time!

4 µl Column Volume
+
13 µl System Volume

5x Equilibration

85 µl

At 30 µl/min, ~3 minutes re-equilibration time

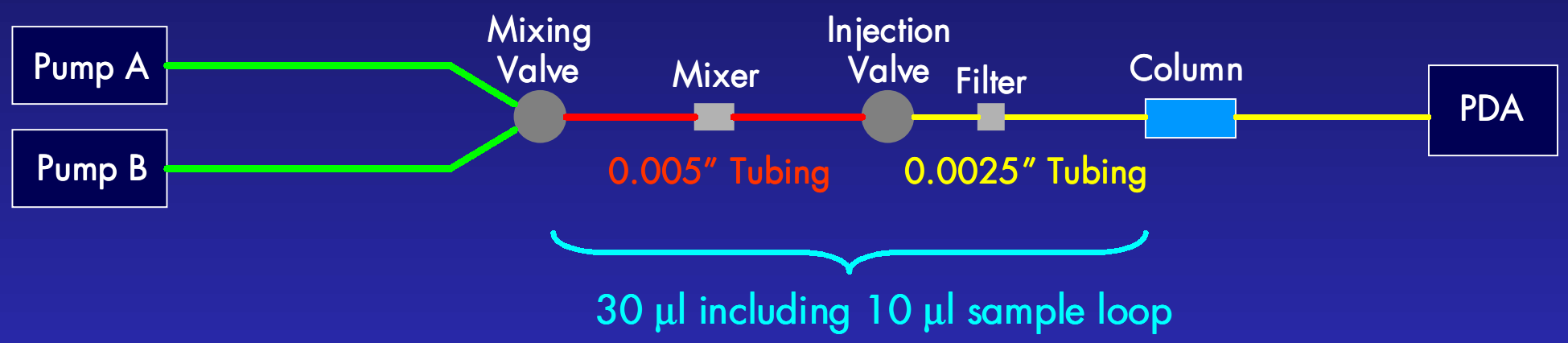
System volume is very important

Minimizing System Volume

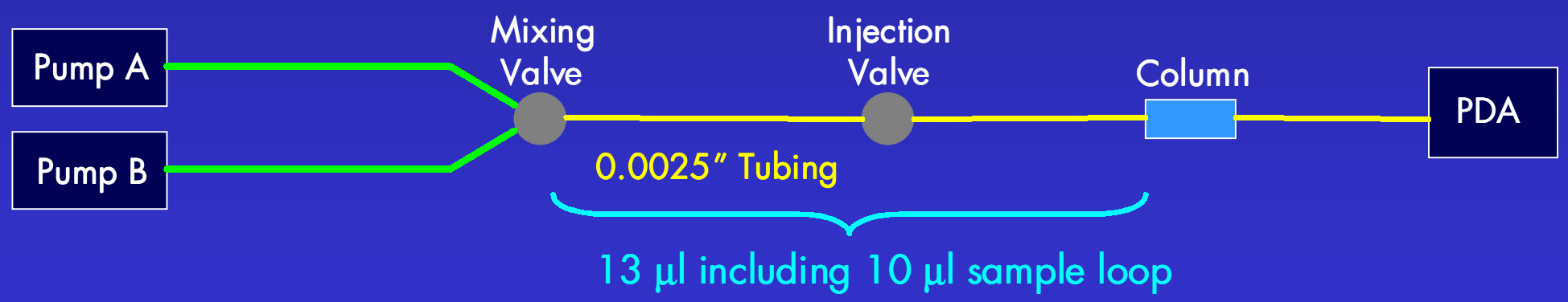
- Reduce internal diameter and length of tubing as much as possible
- Decrease mixing volumes
  - If not working with TFA, mixers can generally be removed from system without greatly affecting baseline or chromatography
    - We have found that removing the mixer when using formic and acetic acid does not deleteriously affect the baseline when using UV detection with a PDA

Capillary LC System Schematic

Standard Plumbing Configuration



Reduced System Volume Configuration



Develop a Generic Gradient Method Appropriate for LC System

- Minimize analysis time by varying
  - Equilibration time
  - System volume
  - Gradient
  - Flow rate
- But…maintain
  - Peak shape
  - Resolution
  - Peak capacity

General Chromatographic Method Parameters

Chromatographic Instrumentation

Waters® CapLC™ System

MS Instrumentation

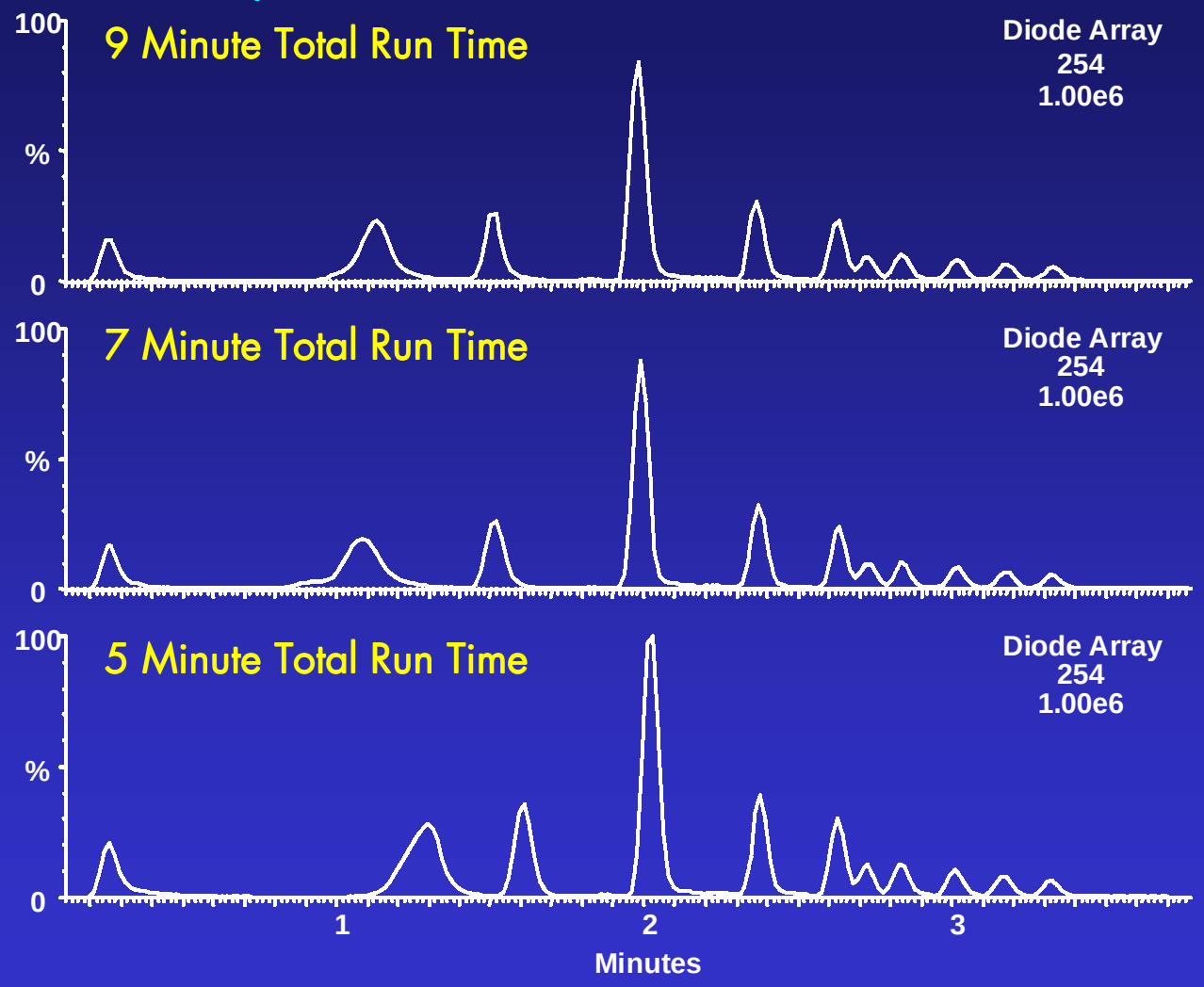
Micromass® Platform LCZ™
Single Quadrupole MS

Chromatographic Parameters

Column: 0.32 x 50 mm and 0.18 x 50 mm
Waters Symmetry® C18, 100 Å, 5 µm
Solvent A: 0.1% formic acid in H_2O
Solvent B: 0.07% formic acid in MeCN
Solvent C: 0.1% formic acid in H_2O
Flow Rates: Varies (7.5-40 µl/min)
Gradient: Varies (5 to 95% B in 2 to 10 min)
Run Time: Varies (2.1 to 25 min)
PDA: 210 to 400 nm

Effect of Equilibration Time

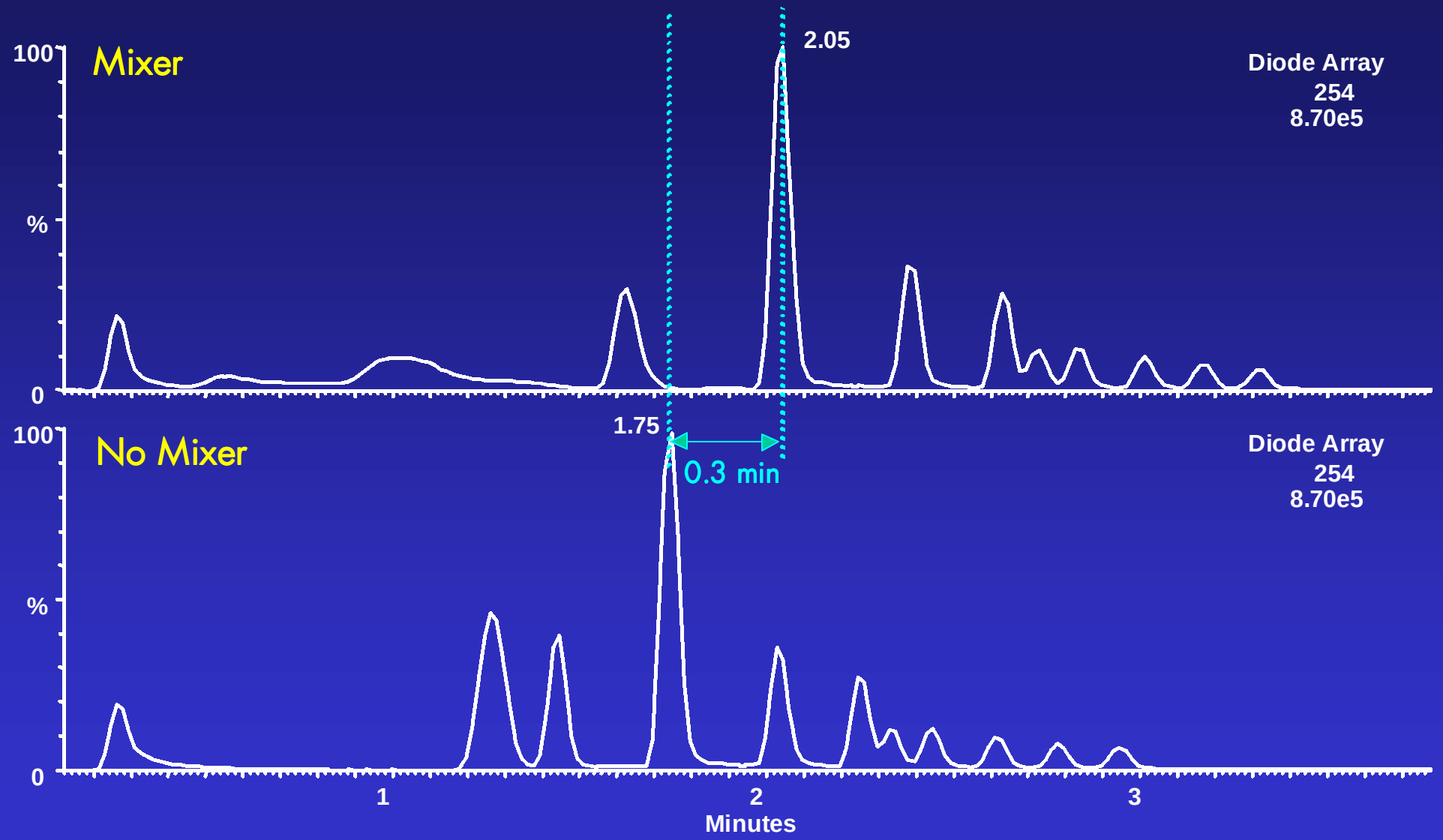
12 Component Mix



- With 5 minute total run time, beginning to see shift in early eluting peaks
- Little change in chromatography of late-eluting peaks with decreasing equilibration time
- Peak capacity for analytes that elute beyond 20-30% organic are not affected by decreasing equilibration time

Gradient: 5 to 95% B in 3 minutes Flow Rate: 30 µl/min With Mixer 30 ng On Column

Reducing System Volume



Gradient: 5 to 95% B in 3 min Flow Rate: 30 µl/min Total Run Time: 4 min 30 ng On Column

Practical Effect of Reducing System Volume

By removing the mixer, the equilibration time decreases:

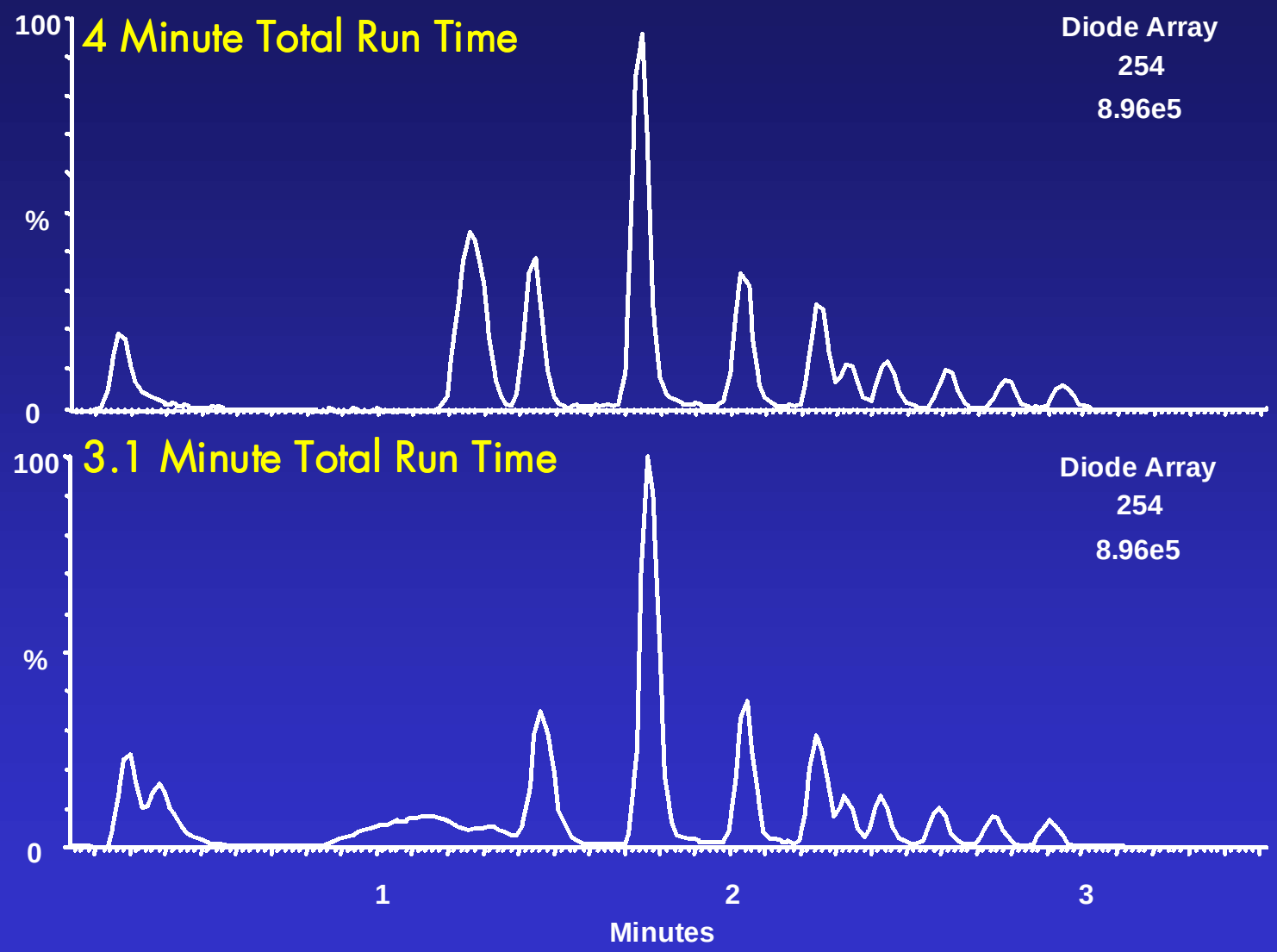
Equilibration Time for System with Mixer

Minus 5 x 0.30 min = 1.5 min

Equilibration Time for System without Mixer

Therefore, can decrease the previously used equilibration time by 1.5 minutes!

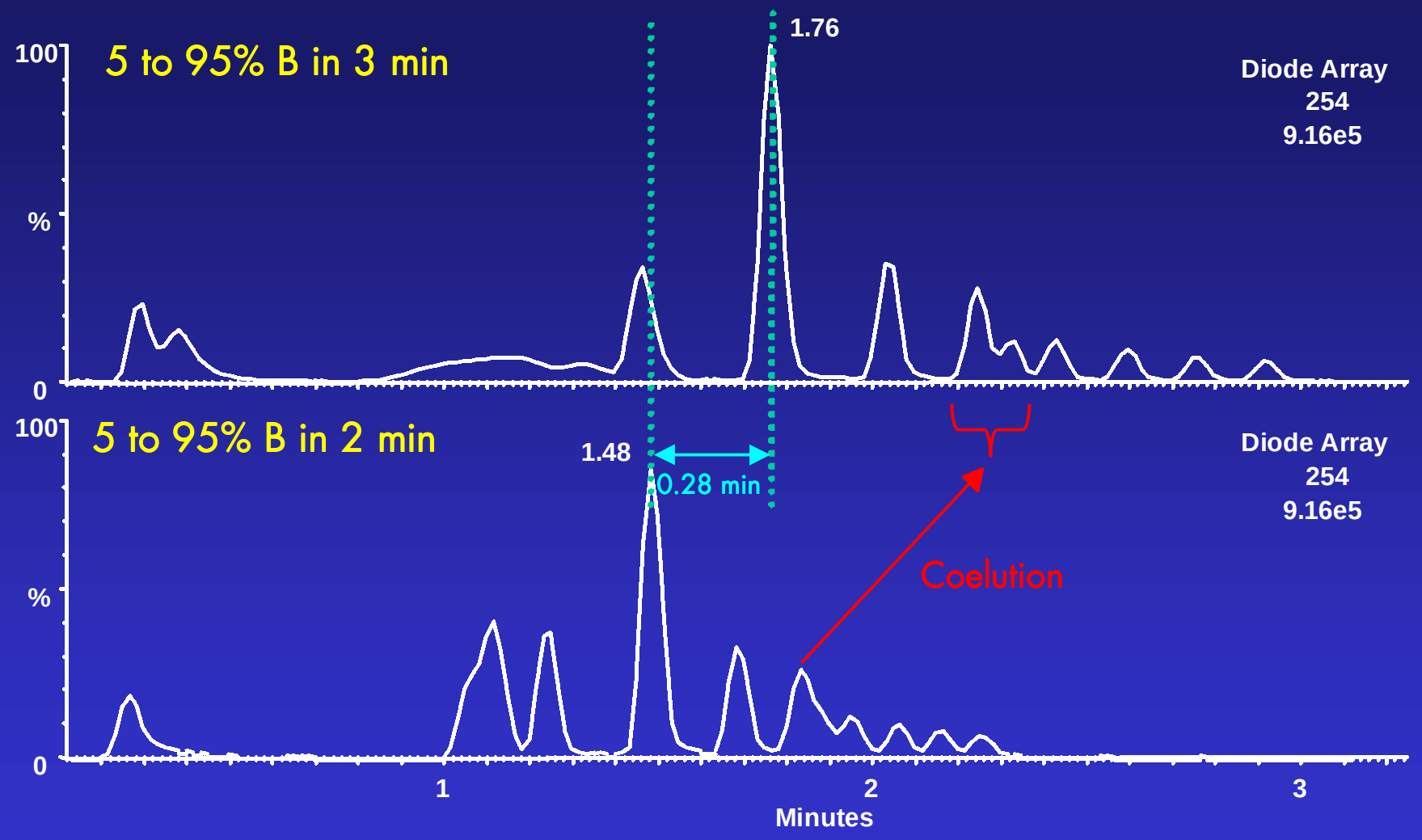
Equilibration Time with No Mixer



- Peaks eluting early in the gradient are affected when total run time is reduced to 3.1 minutes
- Peak capacity and resolution retained for analytes that elute beyond 20-30% organic mobile phase
- If analyzing more hydrophobic compounds, equilibration time can be greatly reduced

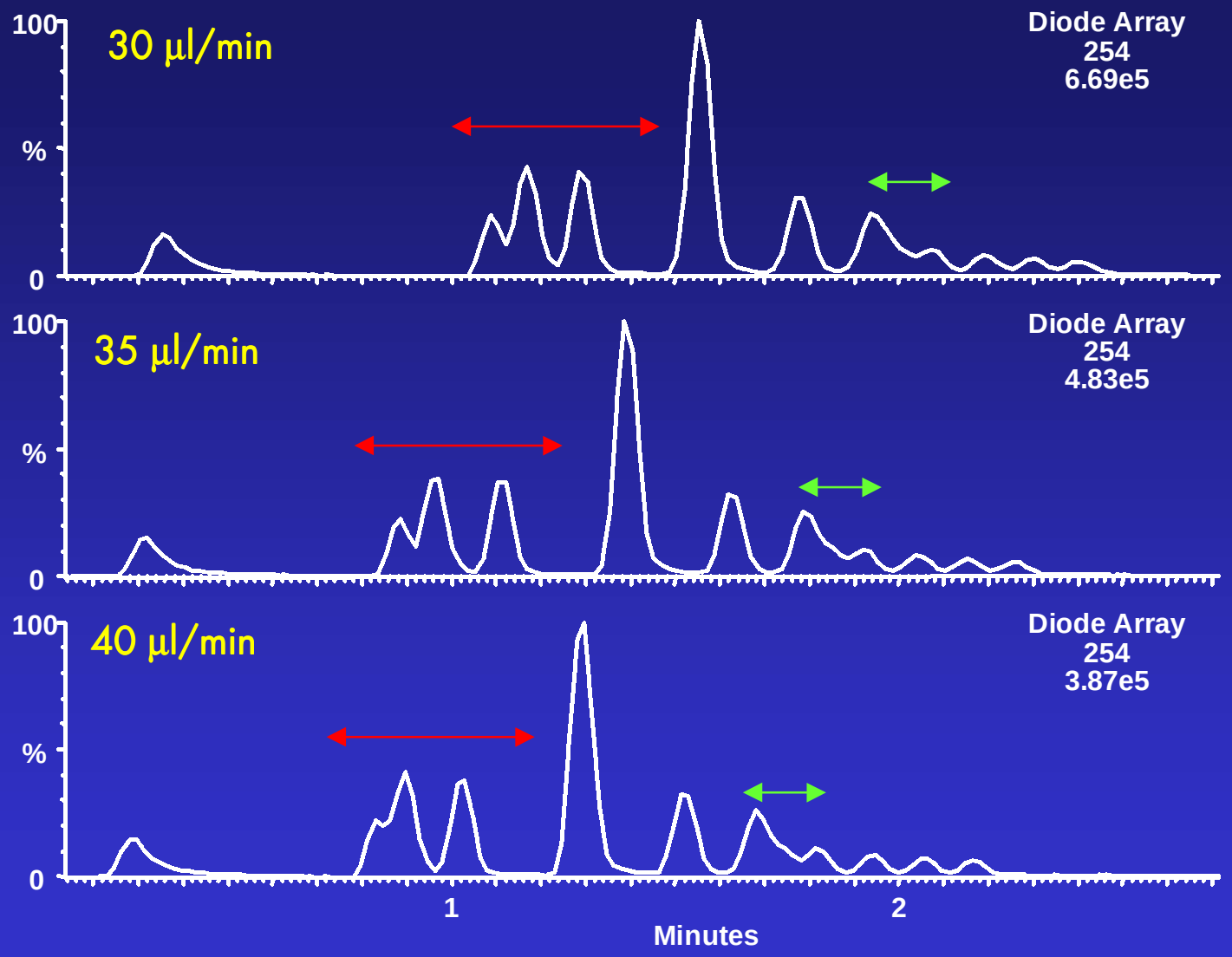
Gradient: 5 to 95% B in 3 minutes Flow Rate: 30 µl/min No Mixer 30 ng On Column

Effect of Gradient



Flow Rate: 30 µl/min Total Run Time: 3.1 min No Mixer 30 ng On Column

Effect of Flow Rate



Loss of efficiency at higher flow rates

Resolution dominated by gradient slope

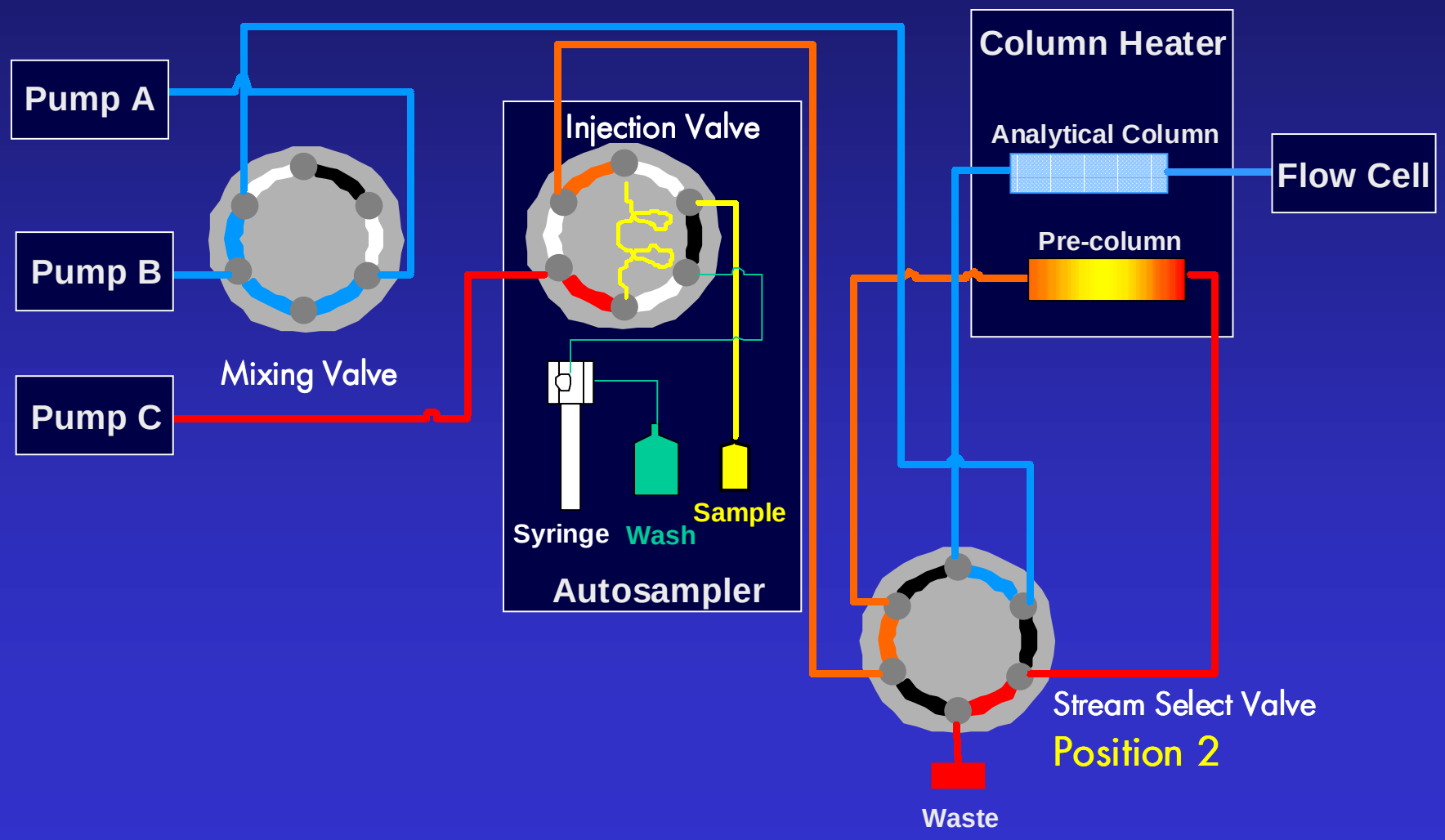
Gradient: 5 to 95% B in 2 minutes Total Run Time: 3.1 min No Mixer 30 ng On Column

Other Considerations

- Sample loading
  - Loading high volumes of sample
  - Loading samples in biological matrices
- Carryover
  - Finding a wash solvent effective for minimizing carryover of analytes of interest

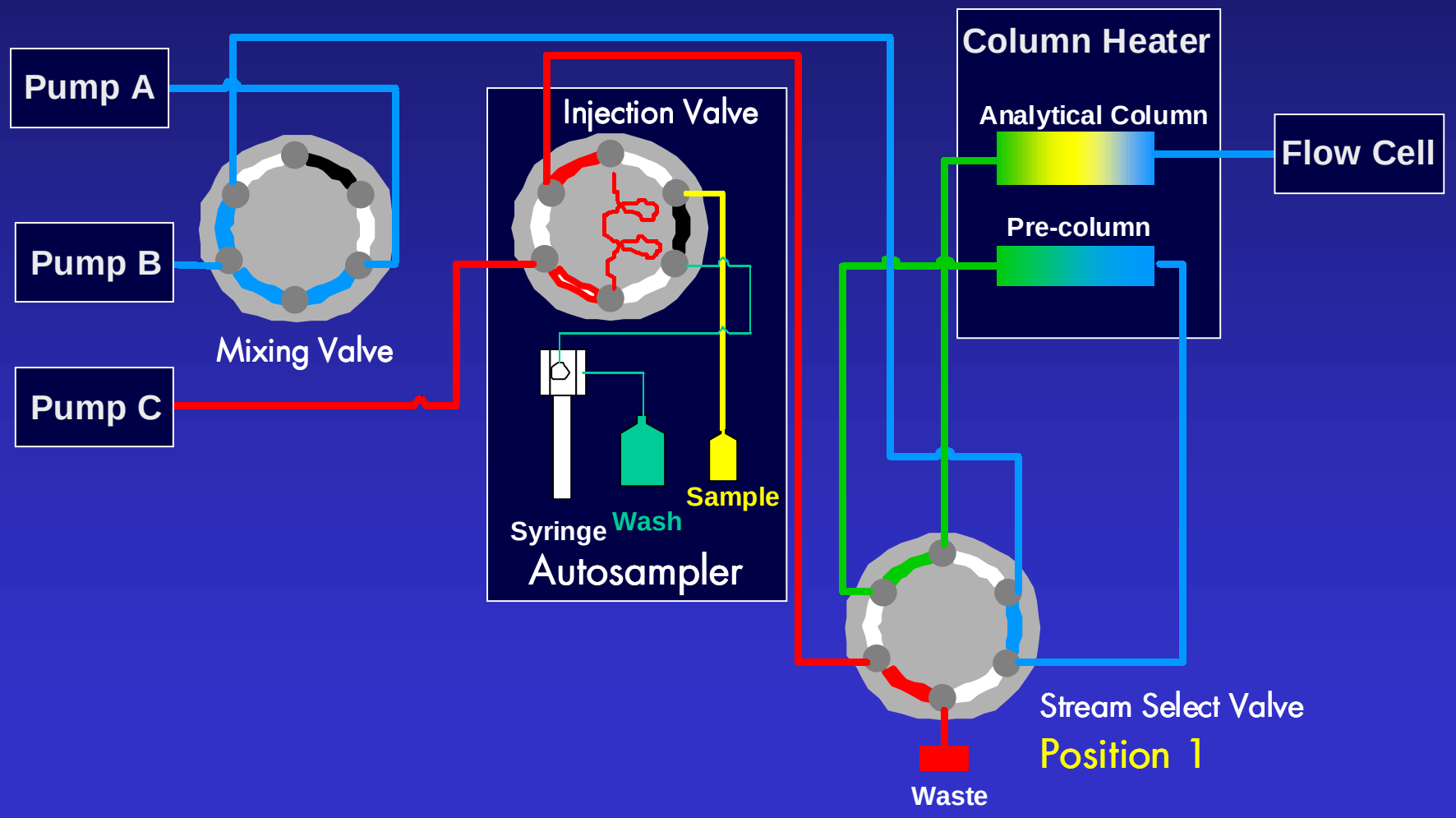
Sample Loading

Sample Trapping Configuration
Loading Sample



Sample Analysis

Sample Trapping Configuration
Eluting Sample



Trapping with a 12 Component Mix

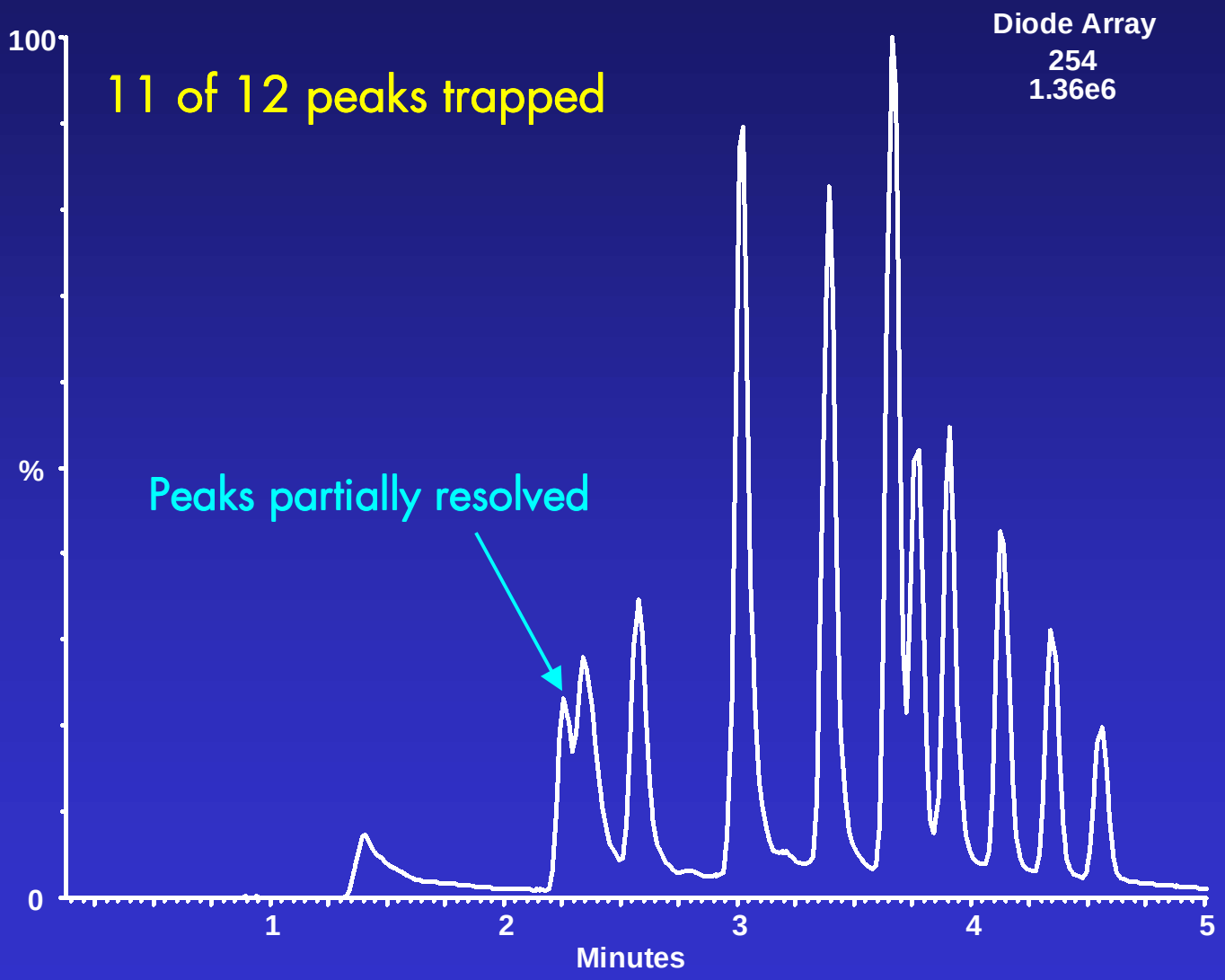
Chromatographic Conditions

Loading Sample

40 µl/min for 1 min
C: 0.1 % formic acid in H_2O
Column: 0.3 x 5 mm C18, 300 Å, 5 µm
Injection Volume: 300 nl
30 ng On Column

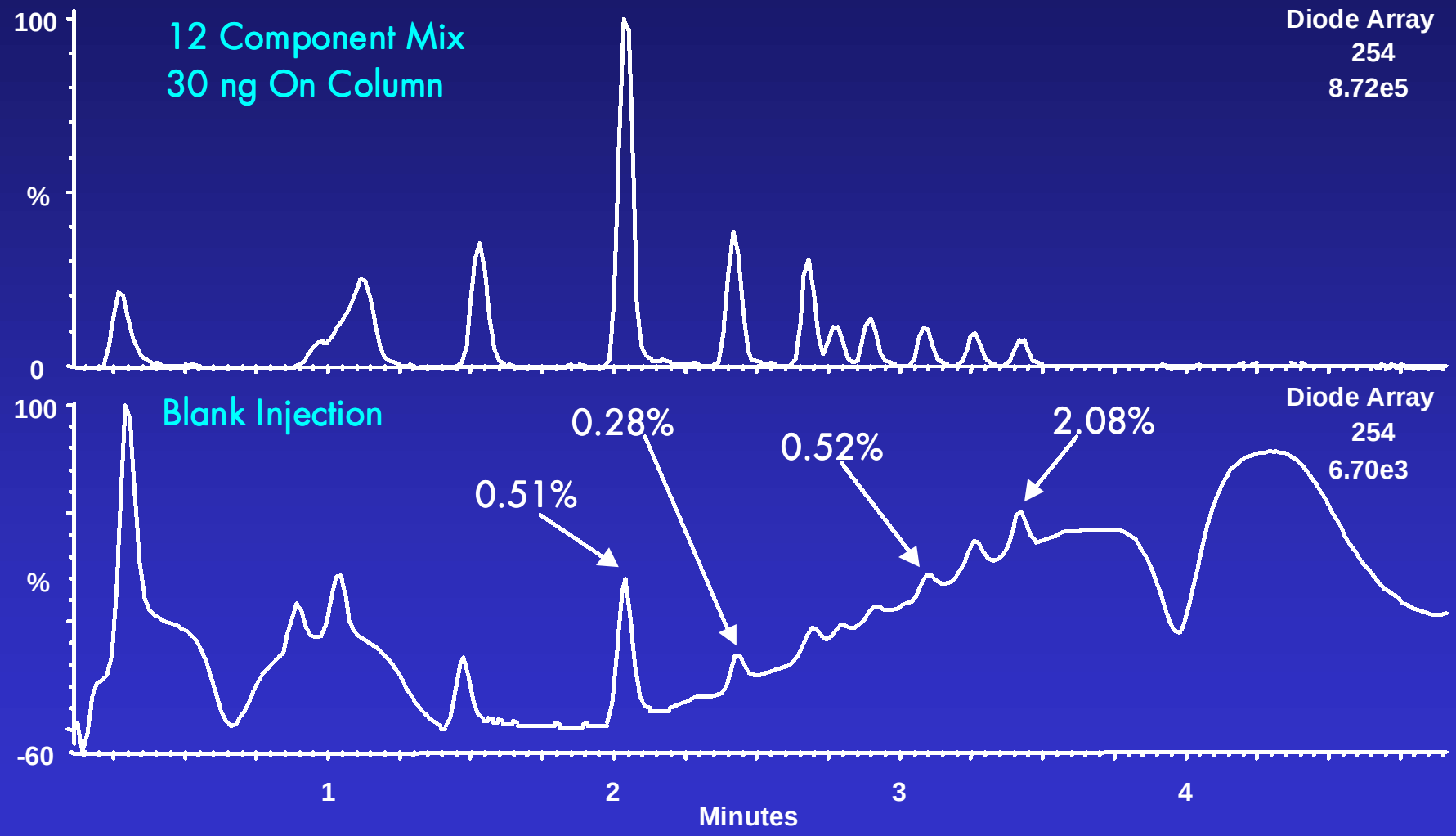
Eluting Sample

Flow Rate: 25 µl/min
A: 0.1% formic acid in H_2O
B: 0.07% formic acid in MeCN
Gradient: 5-95 % B in 4 min
Total Run Time: 8 min
Column: 0.32 x 50 mm Waters Symmetry C18, 300 Å, 5 µm



Carryover

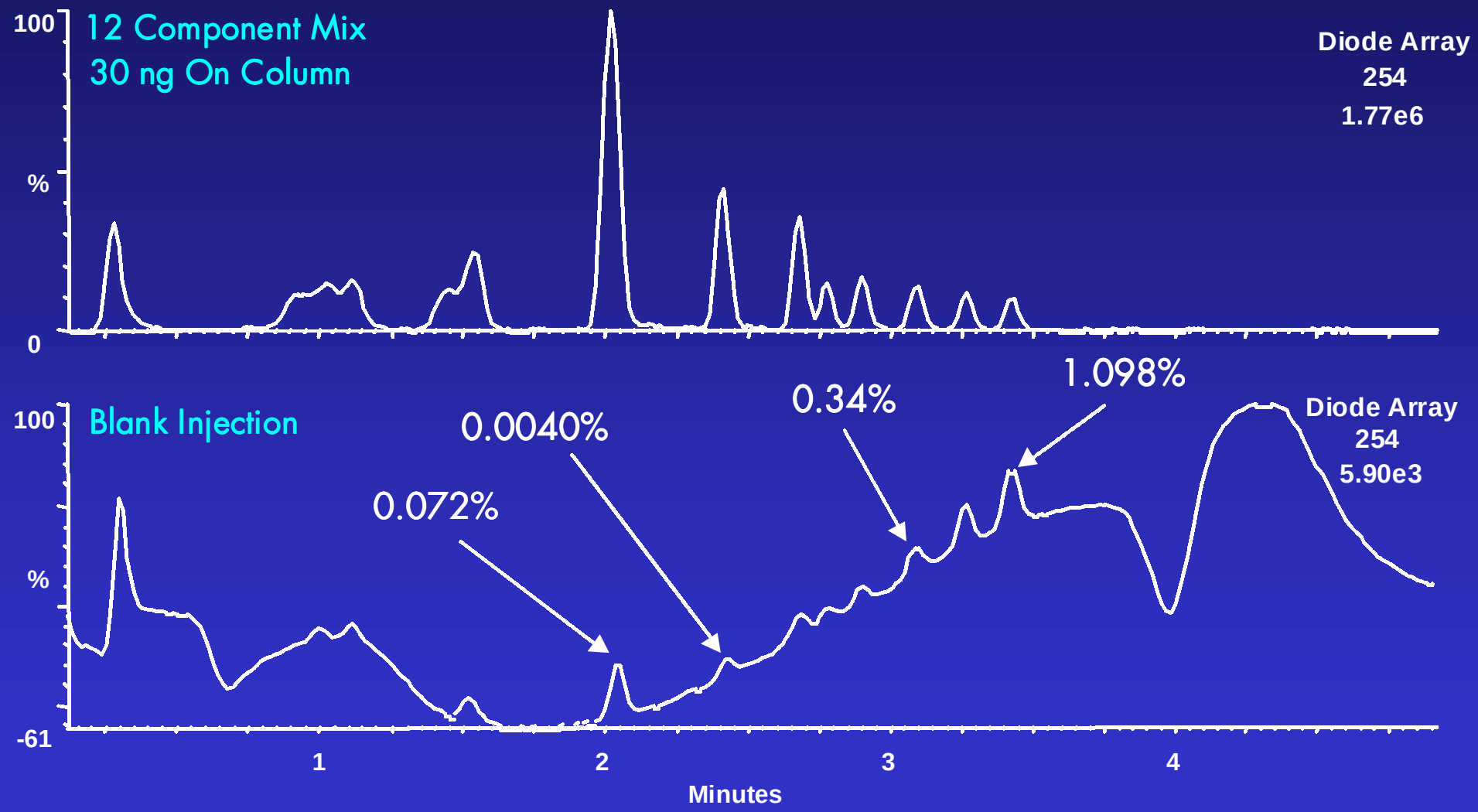
20/80 Methanol/Formic Acid (1%) as Wash Solvent



30 µl/min Gradient: 5 to 95% B in 3 minutes Total Run Time: 5 min

Carryover

80/20 Isopropanol/Water as Wash Solvent

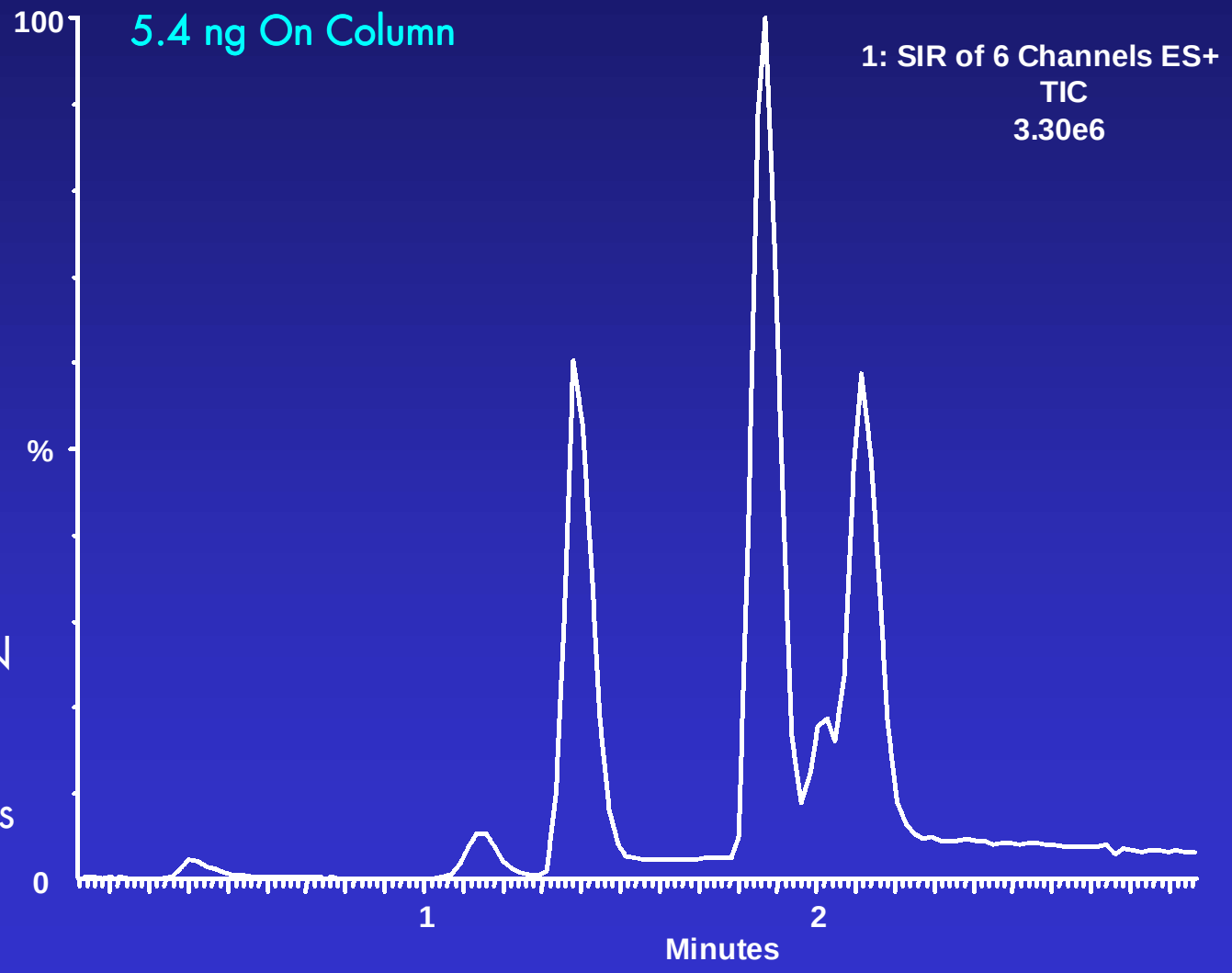


30 µl/min Gradient: 5 to 95% B in 3 minutes Total Run Time: 5 min

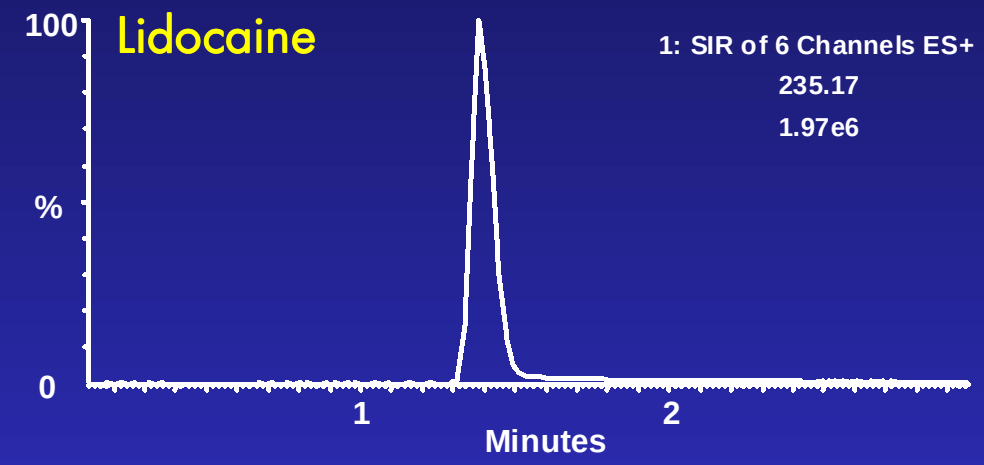
Applying Generic Gradient to Real Samples:
6 Component Drug Mix with LC/MS

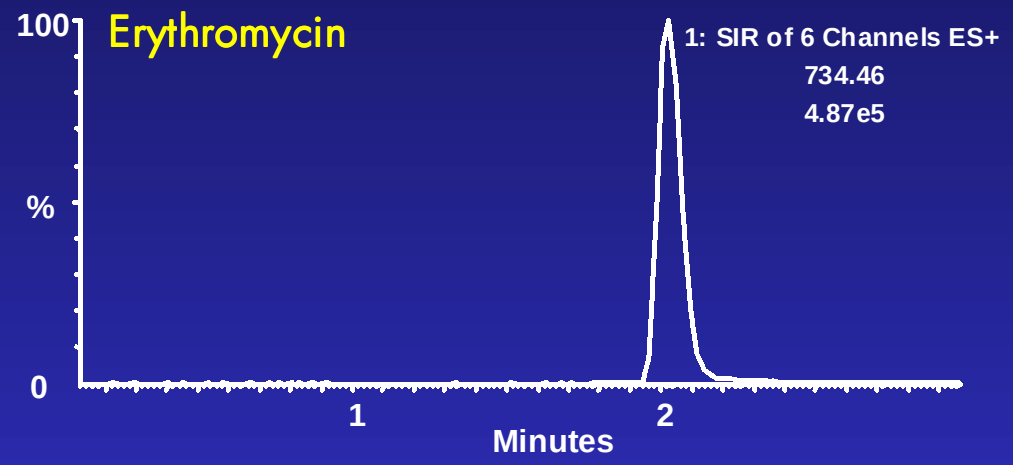
Sample
6 Component Drug Mix
Acetaminophen
Erythromycin
Lidocaine
Na Monensin
Propranolol
Verapamil

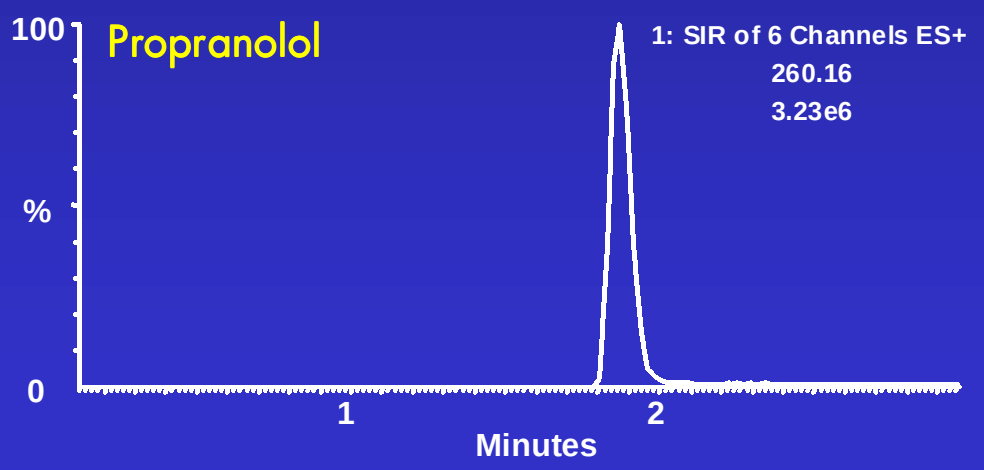
Chromatographic Conditions
Flow Rate: 30 µl/min
A: 0.1% formic acid in H2O
B: 0.07% formic acid in MeCN
Gradient: 5 to 95% B in 3 min
Total Run Time: 5 min
Column: 0.32 x 50 mm Waters Symmetry C18, 300 Å, 5 µm
Injection Volume: 200 nl

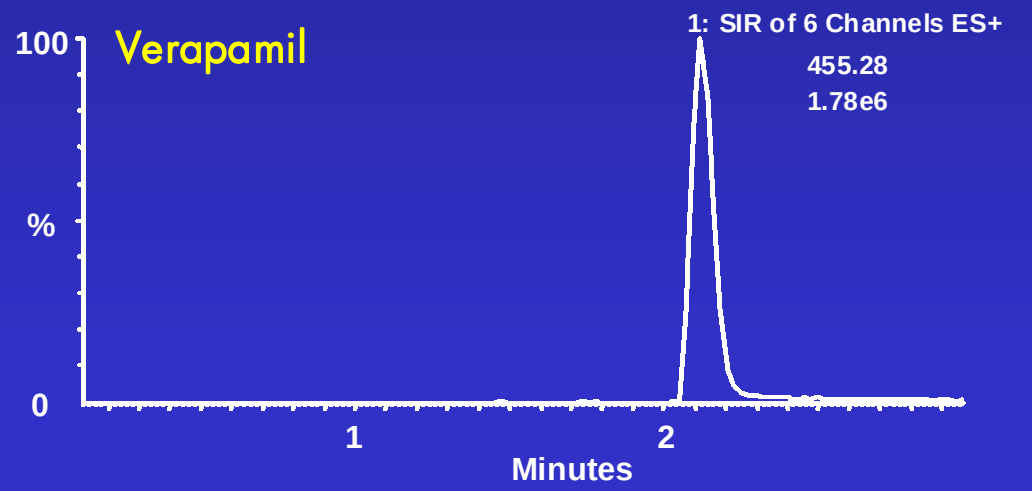


Extracted Ion Chromatograms:
6 Component Drug Mix with LC/MS









Applying Generic Gradient to Real Samples:
5 Component Mix

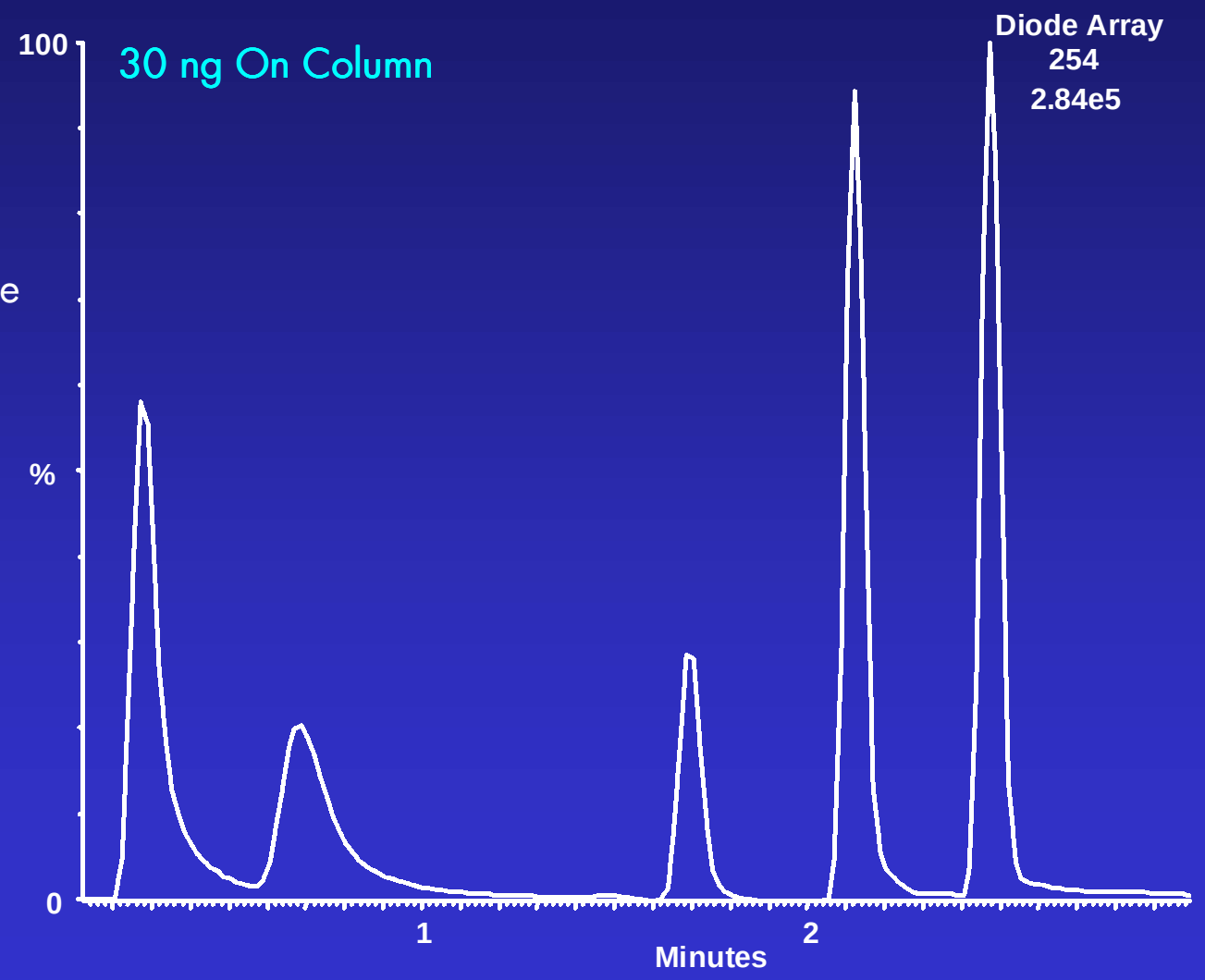
Sample

5 Component Mix (0.1 mg/ml)

uracil
1-hydroxy-7-azabenzatriazole
methyl-3-amino-2-thiophenecarboxylate
4-aminobenzophenone
methoxybenzene sulfonamide

Chromatographic Conditions

Flow Rate: 30 µl/min
A: 0.1% formic acid in H2O
B: 0.07% formic acid in MeCN
Gradient: 5 to 95% B in 3 min
Total Run Time: 5 min
Column: 0.32 x 50 mm Waters Symmetry C18, 300 Å, 5 µm
Injection Volume: 300 nl

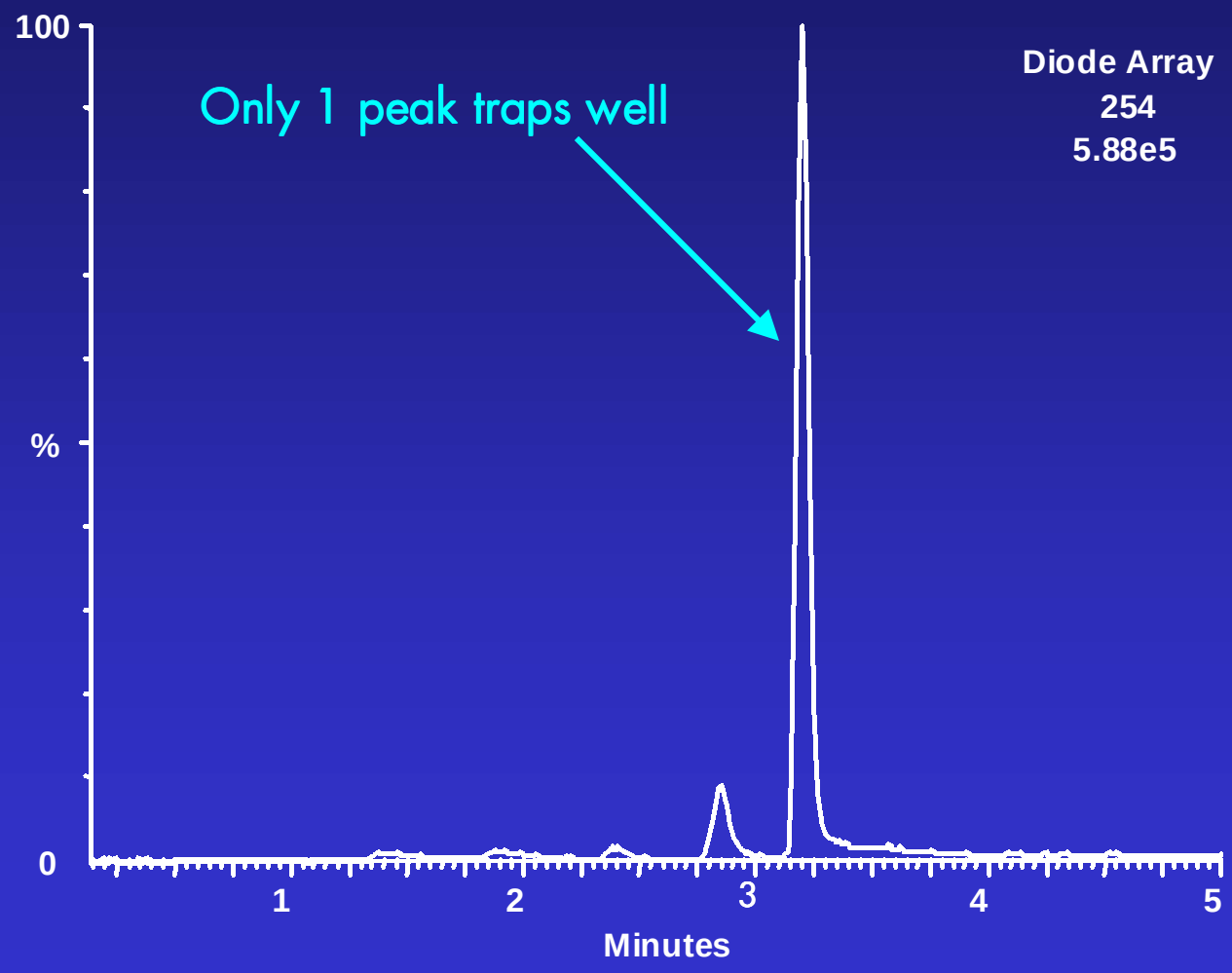


Applying Generic Gradient to Real Samples:
5 Component Mix with Trapping

Chromatographic Conditions

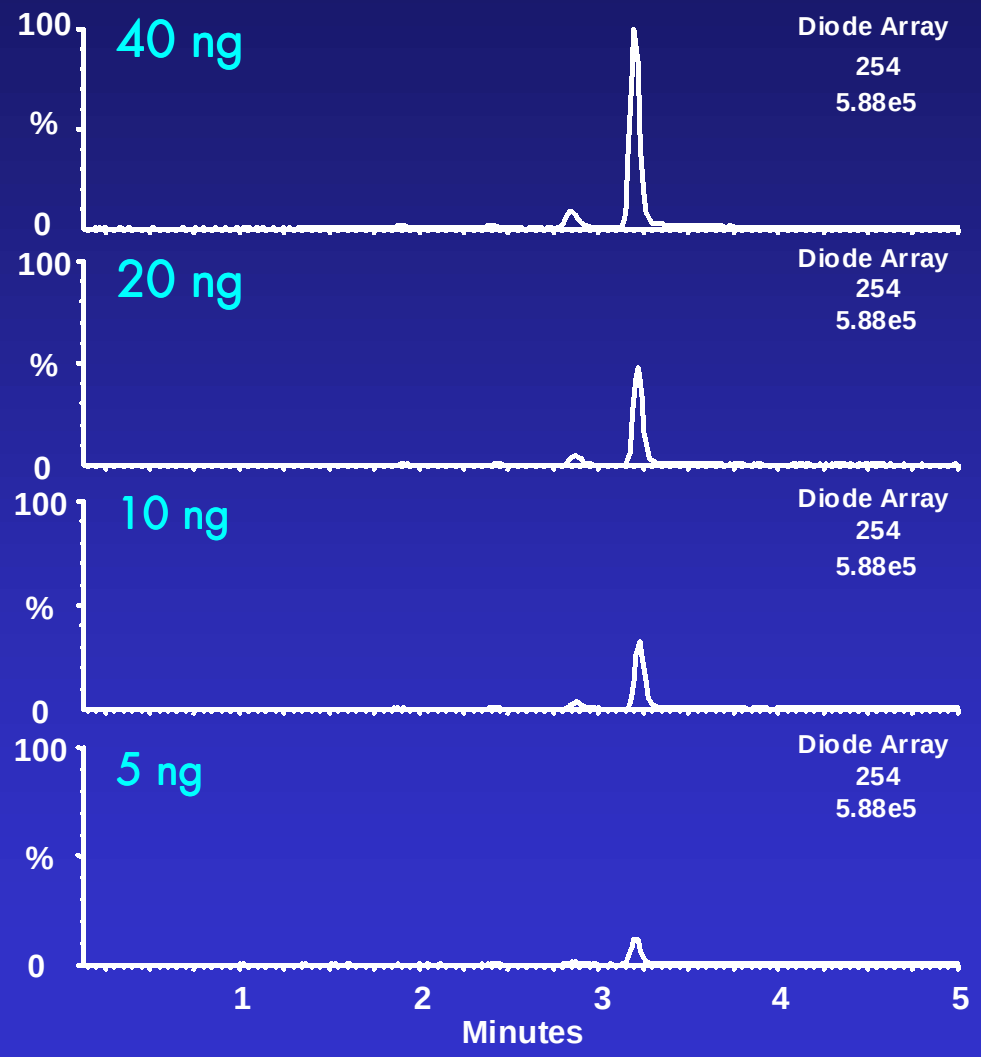
Loading Sample
40 µl/min for 1 min
C: 0.1 % formic acid in H_2O
Column: 0.3 x 5 mm C18, 300 Å, 5 µm
Injection Volume: 300 nl

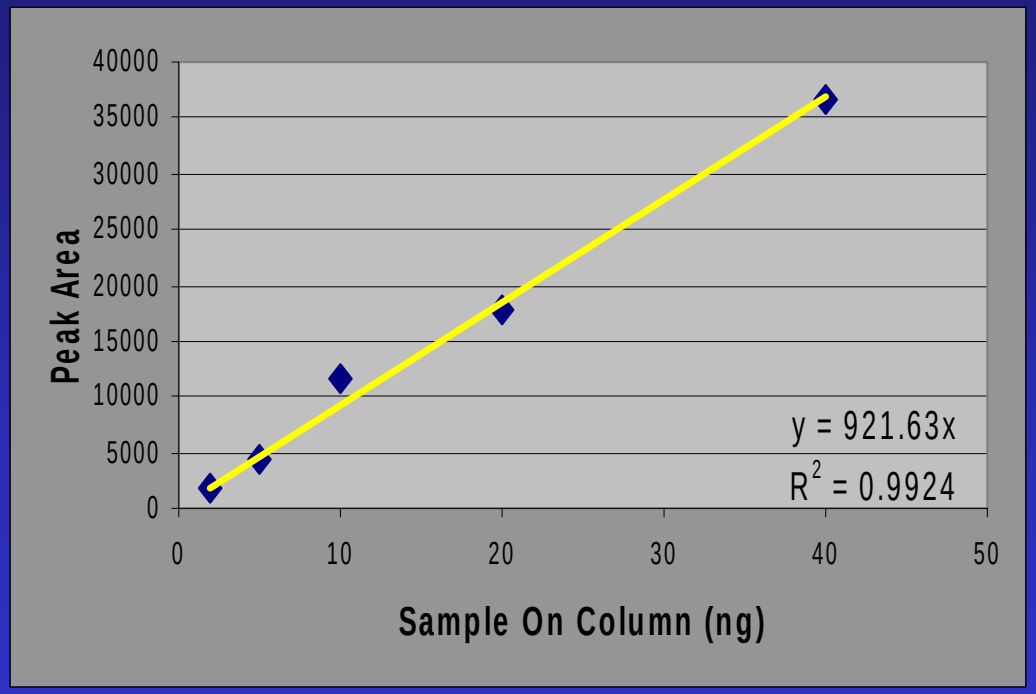
Eluting Sample
Flow Rate: 25 µl/min
A: 0.1% formic acid in H_2O
B: 0.07% formic acid in MeCN
Gradient: 5-95 % B in 4 min
Total Run Time: 8 min
Column: 0.32 x 50 mm Waters Symmetry C18, 300 Å, 5 µm



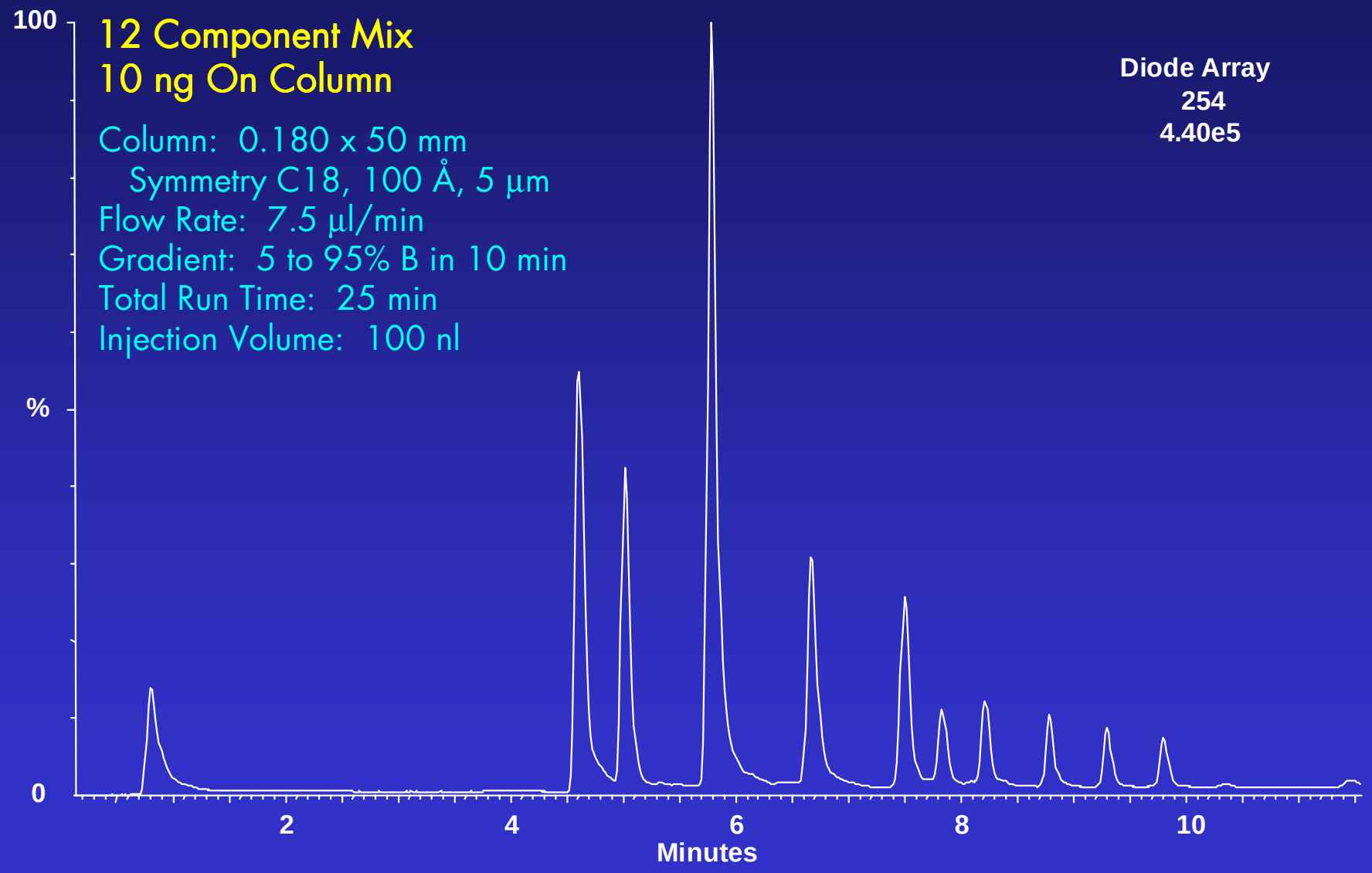
Applying Generic Gradient to Real Samples:

5 Component Mix with Trapping





Enhanced Sensitivity with 180 Micron Column



Summary

- Generic gradients can be developed to minimize analysis time while maximizing peak resolution and capacity
- Analysis time may be greatly reduced if analytes of interest elute beyond 20 to 30% organic mobile phase
- Coeluting compounds can generally be resolved with mass spectrometry

12 Mix Components

Uracil	Acetophenone	Heptanophenone
Theophylline	Propiophenone	Valerophenone
Acetylfuran	Butyrophenone	Octanophenone
Acetanilide	Hexanophenone	Benzophenone