Increasing Sensitivity in HPLC

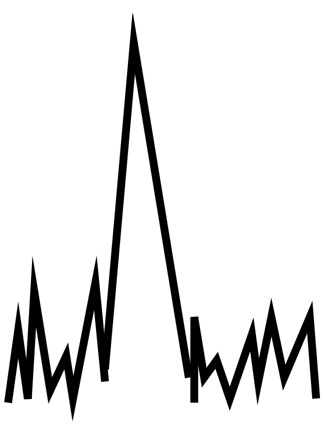
Dr. Shulamit Levin

Medtechnica

levins@medtechnica.co.il

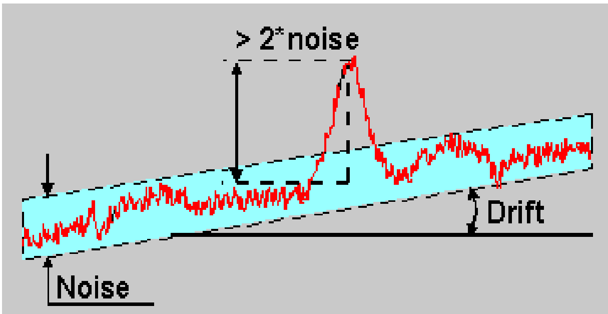
shulal@zahav.net.il

www.forumsci.co.il/HPLC



Noise and drift

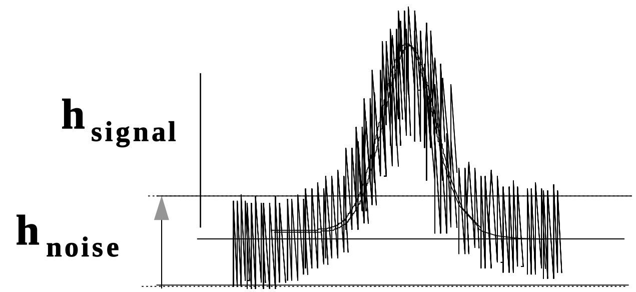
- Noise, drift, and smallest detectable peak.



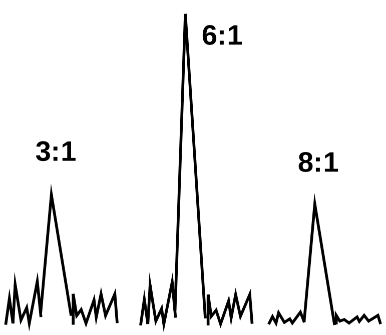
PROPERTIES OF DETECTORS

DETECTION LIMIT

$$h_{signal} = 2 \times h_{noise}$$



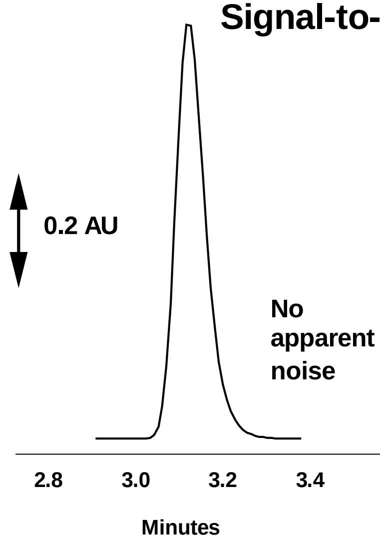
Increase Signal-to-Noise Ratio

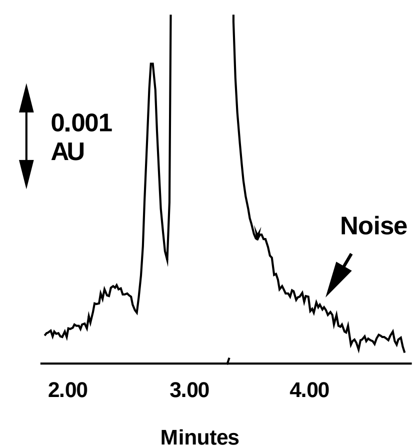


- **Signal-to-noise (S/N) is peak height to noise**
- **Increase S/N by increasing peak height**
- **Increase S/N by decreasing noise**

Chromatographic Sensitivity

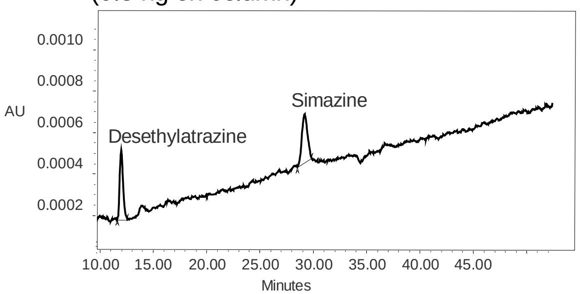
Signal-to-Noise Ratio



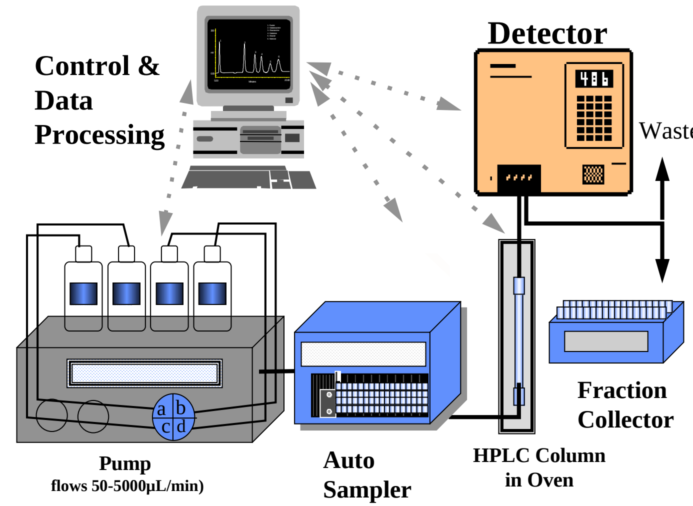


Triazine herbicides at detection limit

Conditions:
Gradient: Phosphate-Acetonitrile
Column Novapak 2 x 300 mm
Sample: 2 ppb each pesticide
Injection: 150 µl
(0.3 ng on column)



HPLC SYSTEM

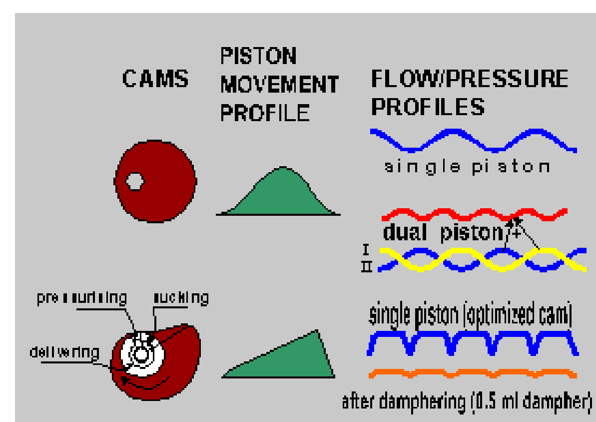


Chromatographic System

- Pump
- Solvent proportioning
- Detector
- Column Perfromance & Geometry
- Stationary phases' chemistry
- Mobile phases' chemistry
- Integration events

Pulse Dampers

- Flow and pressure profiles for different types of pumps and cam shape

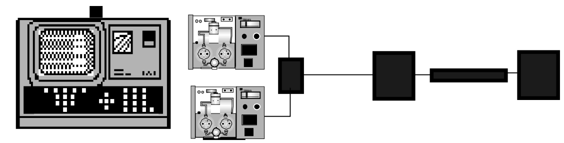


Composition accuracy and consistency

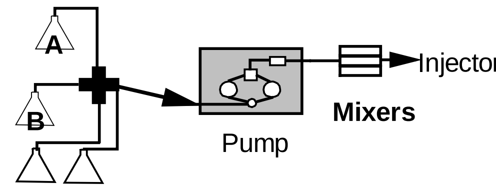
Chromatographic System

- Pump
- Solvent proportioning
- Detector
- Column Perfromance & Geometry
- Stationary phases' chemistry
- Mobile phases' chemistry
- Integration events

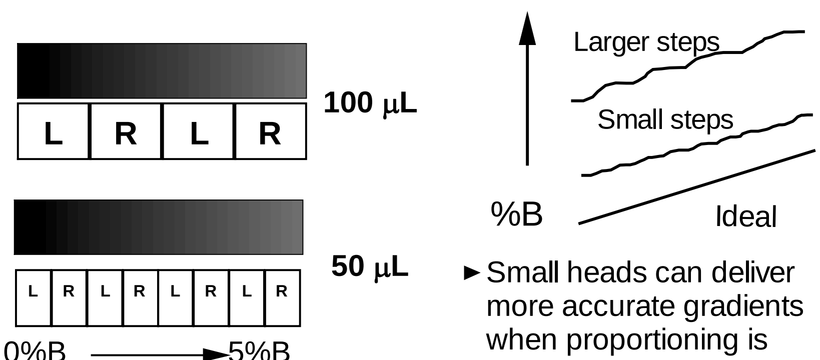
High-Pressure Mixing



Low-Pressure mixing

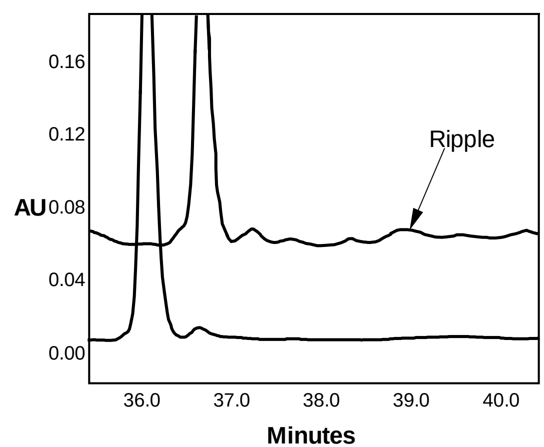


Pump Head Volume



► Small heads can deliver more accurate gradients when proportioning is appropriate (RPS).

Compositional Ripple



- **Older technology**
  - **Baseline "noise" seen (200 nm)**
  - **Insufficient mixing or non-optimized gradient formation**
- **New technology**

Chromatographic System

- Pump
- Solvent proportioning
- Detector
- Column Perfromance & Geometry
- Stationary phases' chemistry
- Mobile phases' chemistry
- Integration events

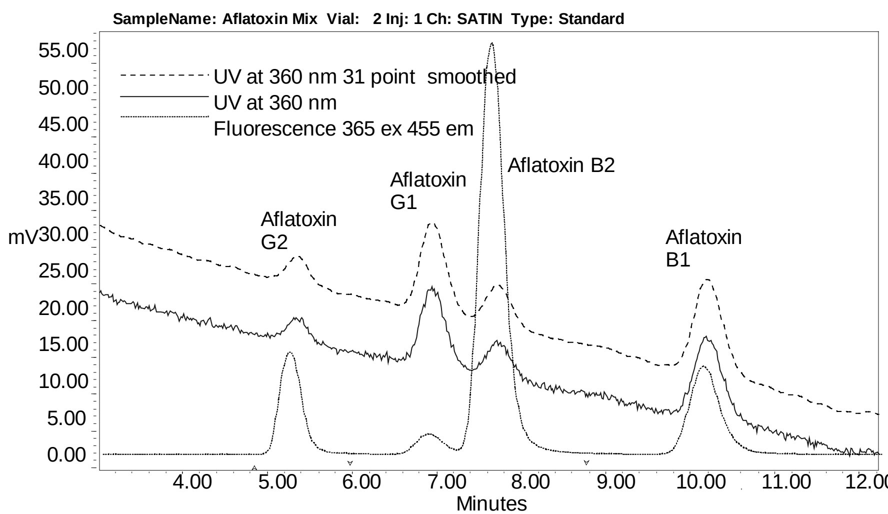
Detectors

- The most common HPLC detectors
  - UV/Vis
    - Fixed wavelength
    - Variable wavelength
    - Diode array
  - Refractive index
  - Fluorescence
  - Electrochemical
-
  - Conductivity
  - Mass-spectrometric (LC/MS)
  - Evaporative light scattering

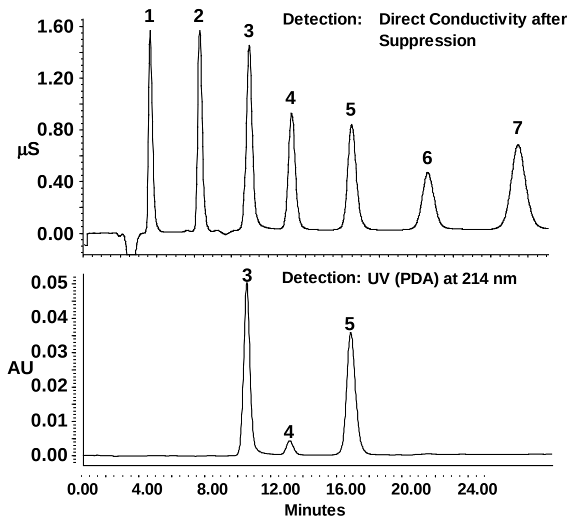
Detector Criteria

- Selectivity
- Sensitivity and detection limit
- Stability
- Linear range
- Dynamic Range
- Reproducibility
- Effect on peak shape
- Maintenance

996 and 474 Comparisons for Aflatoxin Analysis



432 Conductivity and 996 PDA Detectors in Series



1. Fluoride 1 ppm
2. Chloride 2 ppm
3. Nitrite 4 ppm
4. Bromide 4 ppm
5. Nitrate 4 ppm
6. Phosphate 6 ppm
7. Sulfate 4 ppm

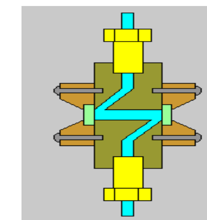
Column:	Waters IC-Pak Anion HR
Eluent:	1.2 mM Sodium Carbonate/ 1.2 mM Sodium Bicarbonate
Flow rate:	1.0 mL/min
Injection vol.:	50 µL

Factors Increasing Signal

- Increase sample concentration
- Increase injection volume
- Choice of wavelength (s)
- Low volume flow cell
- Flow cell pathlength

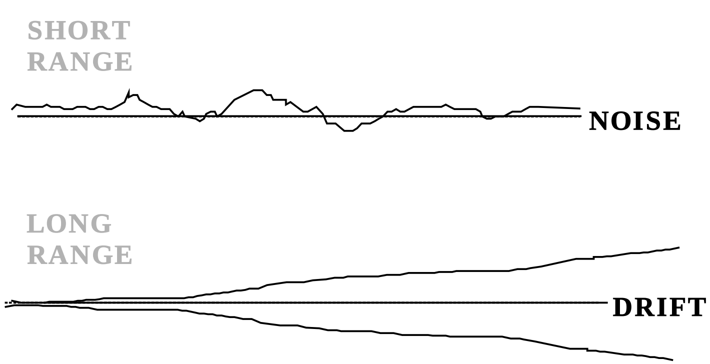
Factors Affecting Noise in HPLC Detectors

- Optics bench design
- Lamp energy
- Wavelengths
- Mobile phase composition
- Pump pulsation
- Electronics



Decrease Noise

BASELINE STABILITY

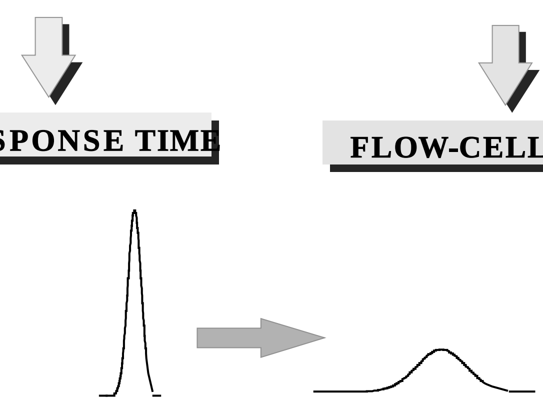


PROPERTIES OF DETECTORS

CONTRIBUTION TO BAND BROADENING

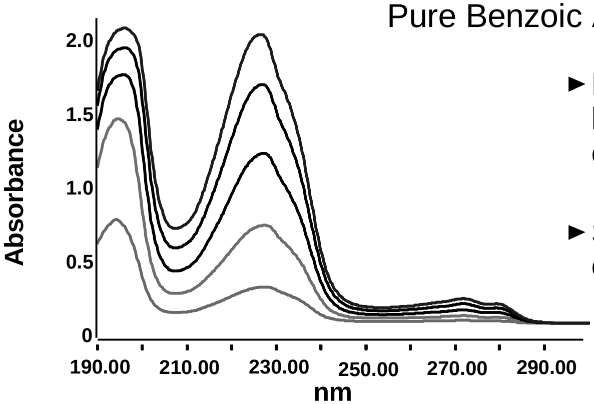
RESPONSE TIME

FLOW-CELL VOLUME



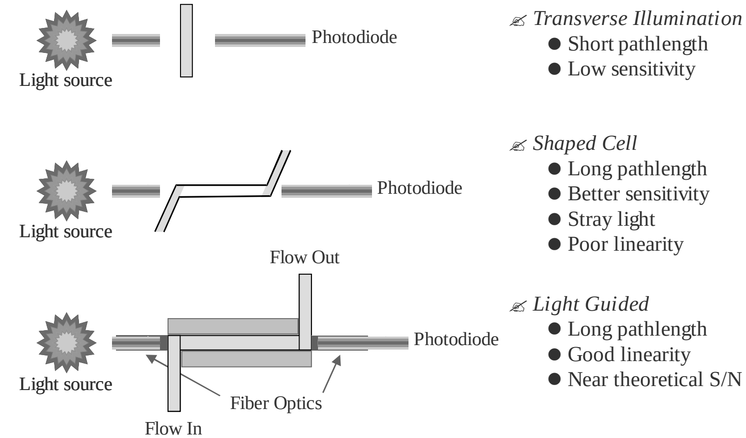
Increase Response
Effect of Concentration on Spectra

Pure Benzoic Acid

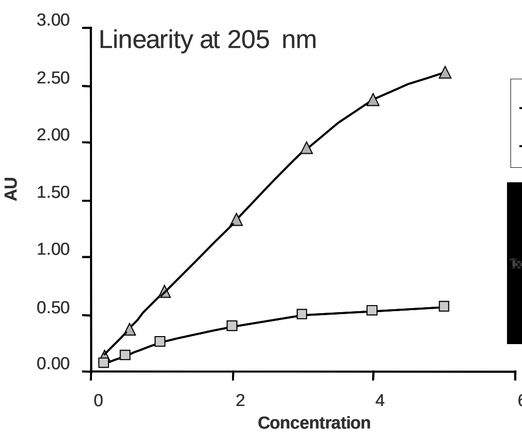


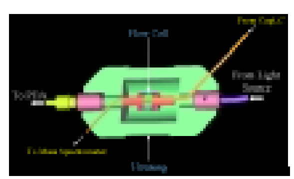
- Different benzoic acid concentrations
- Spectral shape changes

Photodiode Array Detection: Detector Design



Photodiode Array Detection: Flow Cell Performance





Light Guided PDA Flow Cell

Chromatographic System

- Pump
- Solvent proportioning
- Detector
- Column Perfromance & Geometry
- Stationary phases' chemistry
- Mobile phases' chemistry
- Integration events

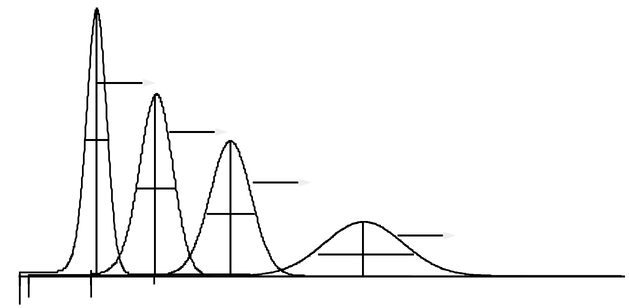
LOQ and LOD Relationship to Column Performances

- LOQ and LOD can be expressed by:

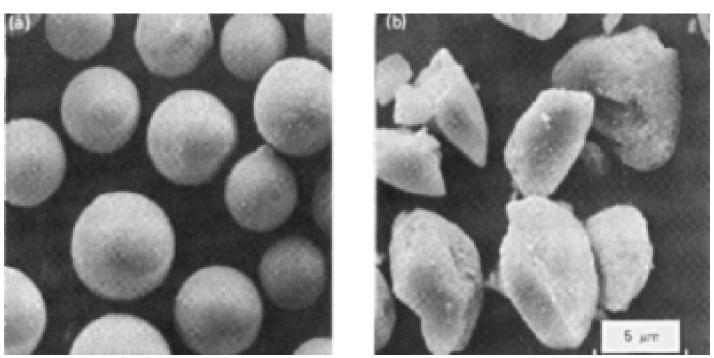
$$C_{max} = \frac{4}{\varepsilon\pi\sqrt{2\pi}} \frac{1}{\left[1+\beta\left(T_a-1\right)\right]} \frac{\sqrt{N}}{L\, d_c^2} \frac{V_{inj}\, c_0}{(1+k')}$$

Peak Shape — **Column Dimension and Performance** — **Retention**

Peak Broadening

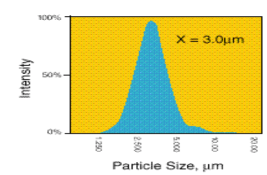


Spherical and Irregular particles

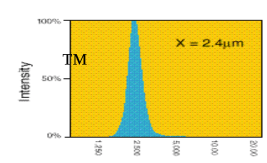


- **Electron microphotograph of spherical and irregular silica particles. [W.R.Melander, C.Horvath, Reversed-Phase Chromatography, in HPLC Advances and Perspectives, V2, Academic Press, 1980]**

Challenge of Making “2 µm” Packings



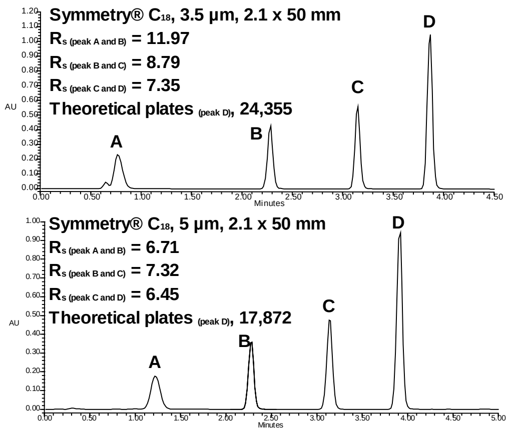
Centered at 3 µm
Wider distribution



Centered at 2.4 µm
Narrower distribution

XTerra COLUMNS

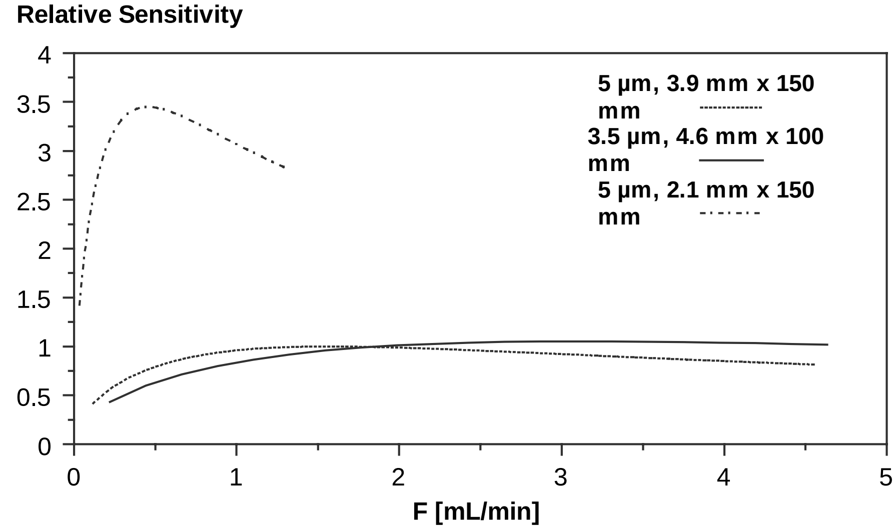
Impact of Particle Size (dp) on Resolution



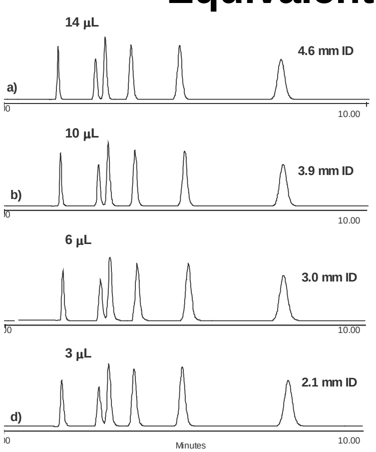
Conditions:

Columns: Symmetry® C_{18}, 5 µm, 4.6 X 50 mm and Symmetry® C_{18}, 3.5 µm, 4.6 X 50 mm
Mobile phase: A=0.1% TFA in water, B=0.1% TFA in acetonitrile
Gradient: 0-60% B in 4 minutes
Column temperature: 30.0 °C
Detector: 254 nm
Injection volume: 1 µL

Sensitivity as a Function of Column Dimensions



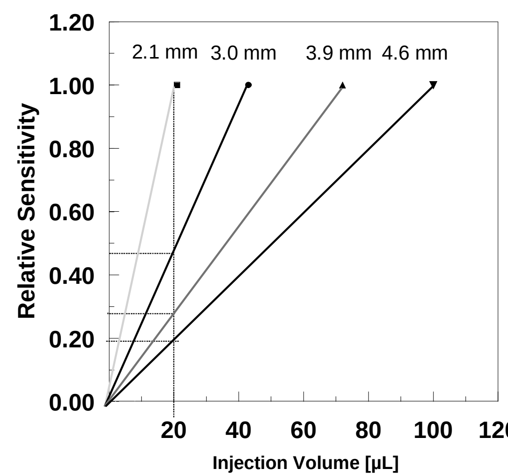
Scaling Injection Volume for Equivalent Resolution



Conditions:	
Columns:	a) Symmetry C_{18} 4.6 mm x 150 mm
	b) Symmetry C_{18} 3.9 mm x 150 mm
	c) Symmetry C_{18} 3.0 mm x 150 mm
	d) Symmetry C_{18} 2.1 mm x 150 mm
Mobile Phase:	water/methanol/glacial acetic acid 79:20:1
Flow Rates:	a) 1.4 ml/min
	b) 1.0 ml/min
	c) 0.6 ml/min
	d) 0.29 ml/min
Sample:	mixture of 6 sulfa drugs

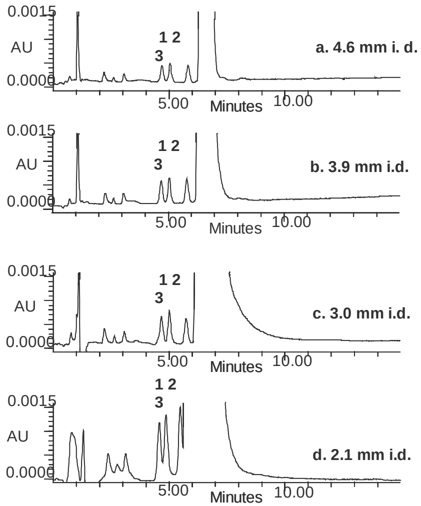
Waters 23,143

Effect of Column Diameter and Injection Volume on Detectability



- k' = 8, N = 10,000
- L = 15 cm

Sensitivity as a Function of Column Diameter



Conditions:

Columns: Symmetry® C_{18} 150 mm length

Mobile Phase: 50 mM potassium phosphate, pH 3.0/acetonitrile 55:45

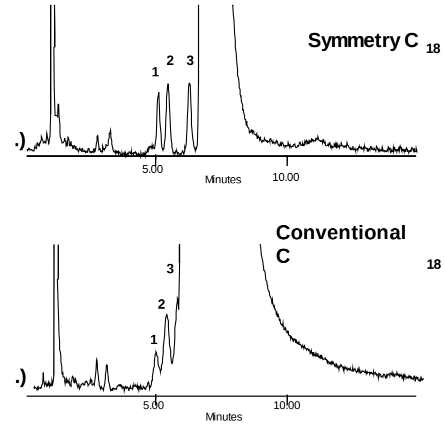
Flow Rates:
- a. 1.4 mL/min
- b. 1.0 mL/min
- c. 0.60 mL/min
- d. 0.29 mL/min

Detection: 240 nm

Sample: tamoxifen, 600 µg/mL, 7 µL injection

	Resolution Peaks 1 and 2	USP Tailing Peak 3
a.)	1.43	1.1
b.)	1.46	1.1
c.)	1.30	1.1
d.)	0.92	n.c.

Tamoxifen: Influence of Asymmetry on Impurity Profile



	USP Plates	Tailing Factors
a)	6500	1.4
b)	1080	3.0

Conditions:

Columns: a) Symmetry C_{18} 3.9 mm x 150 mm; b) Zorbax Rx C_{18} 4.6 mm x 150 mm

Mobile Phase: 50 mM potassium phosphate, pH 3/ acetonitrile 55:45

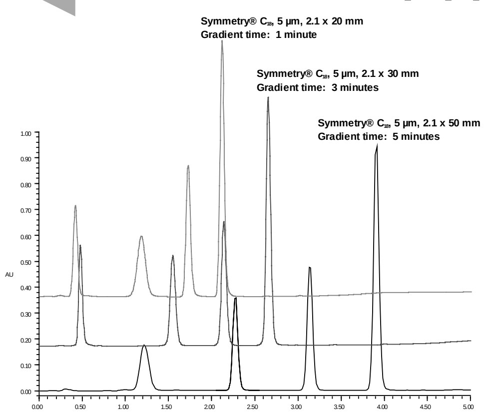
Flow Rates: a) 1.0 ml/min; b) 1.4 ml/min

Detector: 240 nm

Sample: 600 µg/ml, 10 µl injection

Water
23,14

Impact of Column Length on Resolution (Approach 1)



Conditions:

Mobile phase: A=0.1% TFA in water, B=0.1% TFA in acetonitrile
Gradient: 0-60% B in noted time
Column temperature: 30.0 °C
Detector: 254 nm
Injection volume: 1 µL
Flow rate: 1 mL/min.

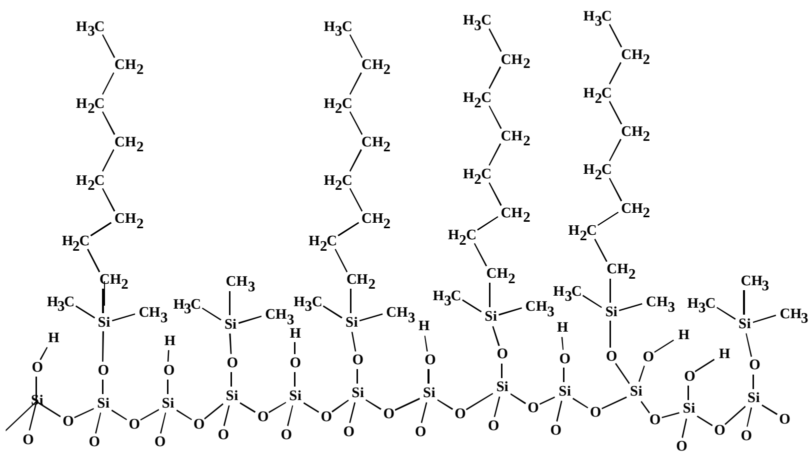
-Maintain resolution when scaling gradient volume proportionally to column volume.

-Reduce analysis time by >50%

Chromatographic System

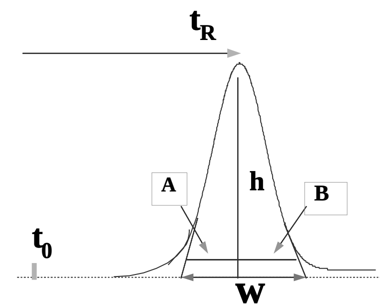
- Pump
- Solvent proportioning
- Detector
- Column Perfromance & Geometry
- Stationary phases' chemistry
- Mobile phases' chemistry
- Integration events

Surface of a Reversed- Phase Packing



PERFORMANCE BY ONE PEAK

NUMBER OF THEORETICAL PLATES

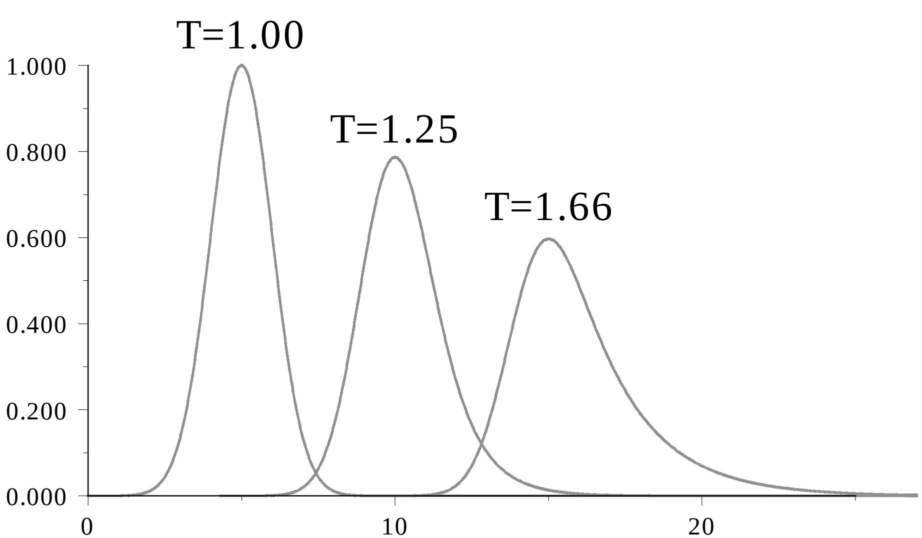


$$N = 16 \left(\frac{t_R}{w}\right)^2$$

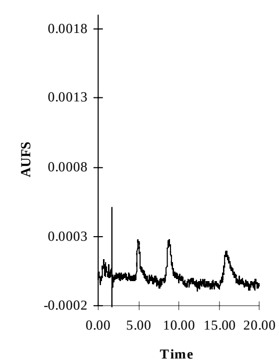
Or:

$$N = 5.54 \left(\frac{t_R}{w_{1/2}}\right)^2$$

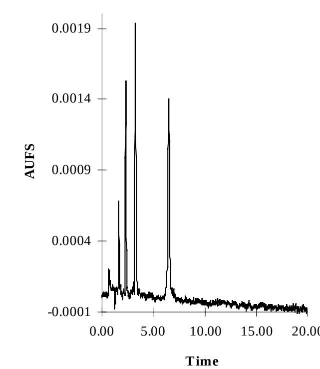
Example of Lowered Peak-Height



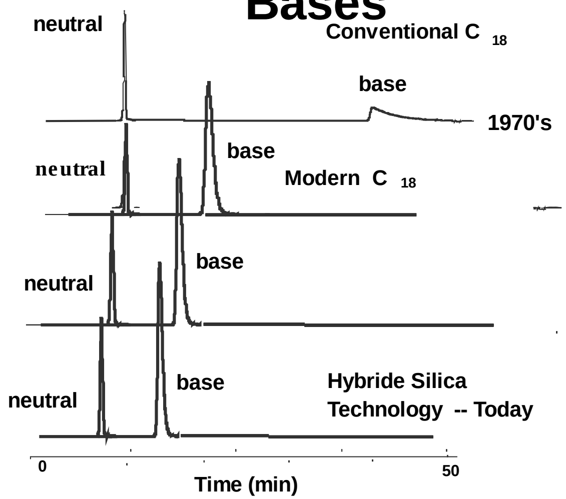
Zorbax 40 nG Procainamide



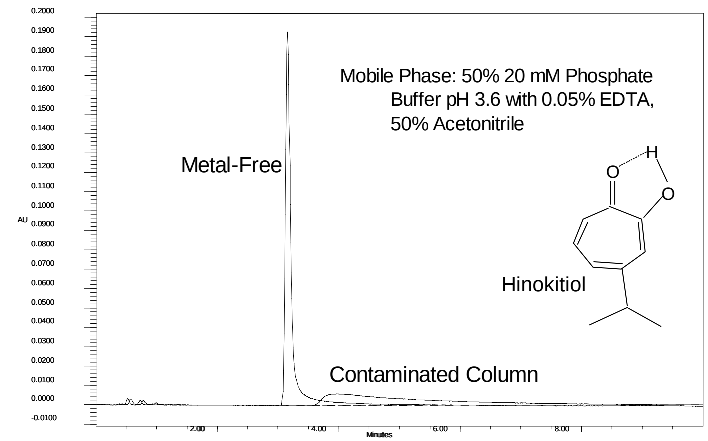
Symmetry 40nG Procainamide



Improvement in Peak Shape for Bases



Peak Forms of Complexing Agent (Hinokitiol)



Chromatographic System

- Pump
- Solvent proportioning
- Detector
- Column Perfromance & Geometry
- Stationary phases' chemistry
- Mobile phases' chemistry
- Integration events

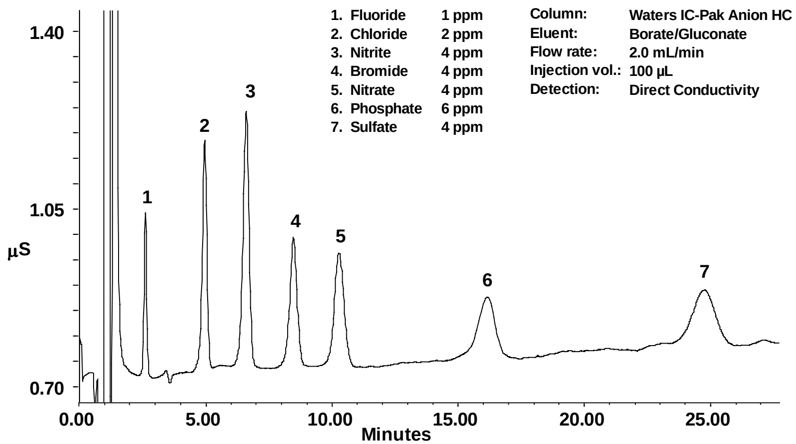
Solvent Effects

Wavelength nm →

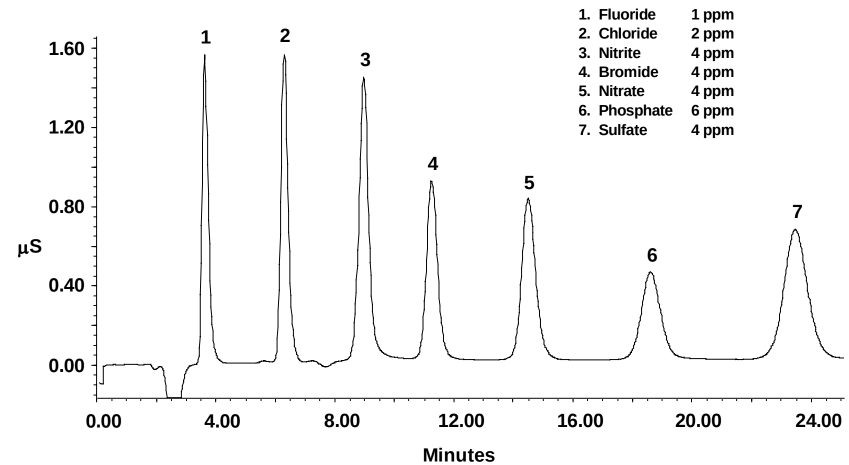
	200	205	210	215	220	230	240	250
Acetonitrile	0.05	0.03	0.02	0.01	0.01	<.01		
Methanol	2.06	1.00	0.53	0.37	0.24	0.11	0.05	0.02
Degassed	1.91	0.76	0.35	0.21	0.15	0.06	0.02	<.01
Isopropyl	1.89	0.68	0.34	0.24	0.19	0.08	0.04	0.03
New THF	2.44	2.57	2.31	1.80	1.54	0.94	0.42	0.21
Old THF	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5
1% HOAc	2.50	2.54	2.47	2.37	2.16	1.01	0.17	0.04
0.1% HPO4	0.01	0.01	0.01	<0.01				
0.1% TFA	1.82	0.87	0.68	0.36	0.22	0.07	<0.01	
1% TEA	2.33	2.42	2.50	2.45	2.37	1.96	0.50	0.12

← Absorbance AU

432 Conductivity Detection of Seven Anion Standard

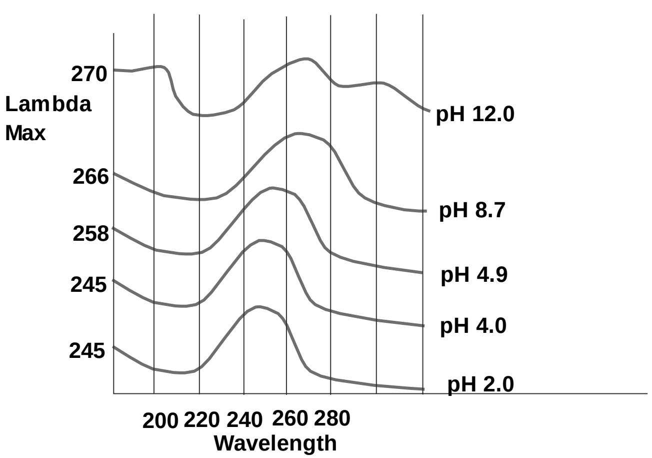


432 Conductivity Detection of Seven Anions with Chemical Suppression



Basic HPLC Review

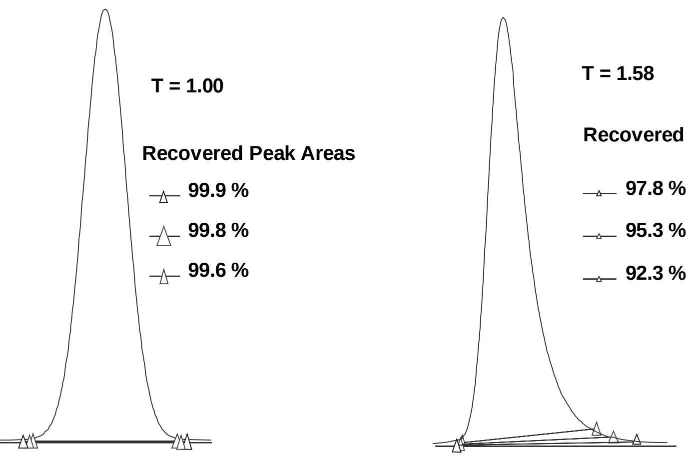
pH Effects on UV Spectra (L-Ascorbic Acid)



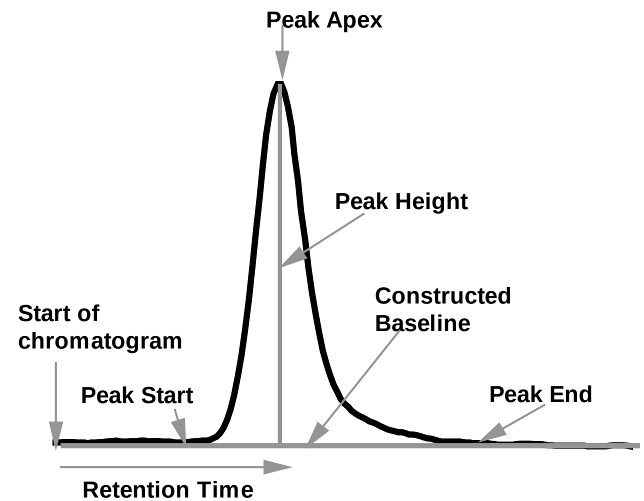
Chromatographic System

- Pump
- Solvent proportioning
- Detector
- Column Perfromance & Geometry
- Stationary phases' chemistry
- Mobile phases' chemistry
- Integration events

Integration Errors Caused by Tailing

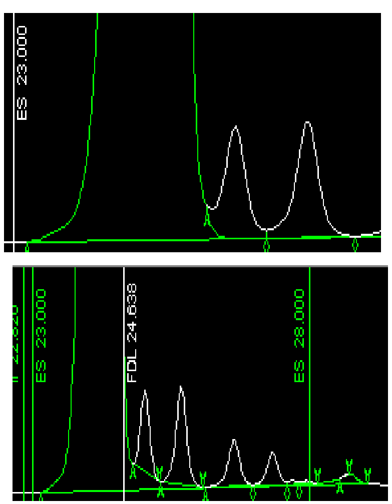


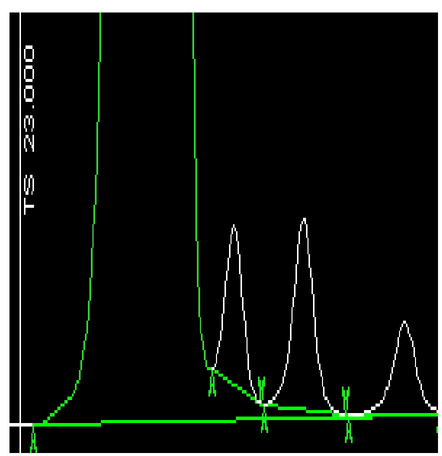
Peak Detection:



Exponential Skim   Tangential Skim

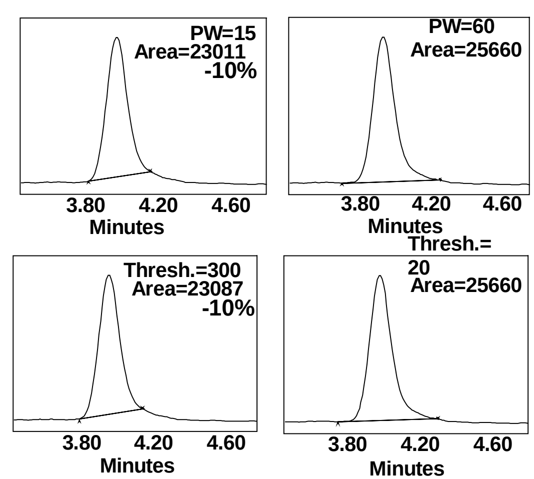
Resolves rider or shoulder peaks from a parent peak. ý





The Tangential and Exponential Skim events require the use of aþ þForce Drop Line within the parent peak. For best results, add onlyýþ þ a single drop line per peak that is to be skimmedýþ .

Integration of Small Peaks



- AUFS = 0.003
- Peak width changed
- Threshold set at 30

- AUFS = 0.003
- Peak width set at 30 sec
- Threshold changed

Dr. Shulamit Levin, Medtechnica