Increasing Sensitivity in HPLC

Dr. Shulamit Levin

www.forumsci.co.il/HPLC
Noise and drift

- Noise, drift, and smallest detectable peak.

PROPERTIES OF DETECTORS

DETECTION LIMIT

\[ h_{\text{signal}} = 2 \times h_{\text{noise}} \]

\[ h_{\text{signal}} \]

\[ h_{\text{noise}} \]

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

Minutes

0.2 AU

No apparent noise

0.001 AU

Noise
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)

**Chromatographic System**
- Pump
- Solvent proportioning
- Detector
- Column Performance & Geometry
- Stationary phases' chemistry
- Mobile phases' chemistry
- Integration events

**Pulse Dampers**
- Flow and pressure profiles for different types of pumps and cam shape
Chromatographic System

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Pump Head Volume

100 µL

L R L R

Larger steps

Small steps

Ideal

50 µL

L R L R L R

%B

5% B

Composition accuracy and consistency

High-Pressure Mixing

Low-Pressure mixing

Composition ripple

Larger steps

Small steps

Ideal

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

New technology

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

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

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

996 and 474 Comparisons for Aflatoxin Analysis

SampleName: Aflatoxin Mix Vial: 2 Inj: 1 Ch: SATIN Type: Standard

[Graph showing UV and fluorescence detection for aflatoxins]
432 Conductivity and 996 PDA Detectors in Series

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

SHORT RANGE

NOISE

LONG RANGE

DRIFT

PROPERTIES OF DETECTORS
CONTRIBUTION TO BAND BROADENING

RESPONSE TIME

FLOW-CELL VOLUME

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Increase Response
Effect of Concentration on Spectra

Pure Benzoic Acid

- Different benzoic acid concentrations
- Spectral shape changes

Photodiode Array Detection: Flow Cell Performance

Photodiode Array Detection: Detector Design

Transverse Illumination
- Short pathlength
- Low sensitivity

Shaped Cell
- Long pathlength
- Better sensitivity
- Stray light
- Poor linearity

Light Guided
- Long pathlength
- Good linearity
- Near theoretical S/N

Chromatographic System

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LOQ and LOD Relationship to Column Performances

LOQ and LOD can be expressed by:

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

Peak Shape

Column Dimension and Performance

Retention

Spherical and Irregular particles


Challenge of Making “2 µm” Packings

Centered at 3 µm
Wider distribution

Centered at 2.4 µm
Narrower distribution

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Impact of Particle Size (dp) on Resolution

Symmetry® C<sub>18</sub>, 3.5 µm, 2.1 x 50 mm
R<sub>p</sub> (peak 1): 11.97
R<sub>p</sub> (peak 2): 6.79
Theoretical plates: 24,355

Symmetry® C<sub>18</sub>, 5 µm, 2.1 x 50 mm
R<sub>p</sub> (peak 1): 7.32
R<sub>p</sub> (peak 2): 6.45
Theoretical plates: 17,872

Scaling Injection Volume for Equivalent Resolution

Conditions:
Columns: Symmetry® C<sub>18</sub>
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

Concentration of Particle Size (dp)

Relative Sensitivity

Effect of Column Diameter and Injection Volume on Detectability

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

Waters 12,143
**Sensitivity as a Function of Column Diameter**

- a. 4.6 mm i.d.
- b. 3.9 mm i.d.
- c. 3.0 mm i.d.
- d. 2.1 mm i.d.

**Conditions:**
- Columns: Symmetry® C 18150 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
tamoxifen, 600 pg/mL
7 mL injection

**Resolution Peaks 1 and 2**
- a.) 1.43
- b.) 1.46
- c.) 1.30
- d.) 0.92

**USP Tailing Peak 3**
- a.) 1.1
- b.) 1.1
- c.) 1.1
- d. n.c.

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**Tamoxifen: Influence of Asymmetry on Impurity Profile**

**Symmetry® C 18**
- USP Plates: 6500
- Tailing Factors: 1.4

**Conventional C 18**
- Conditions:
  - Columns: a) Symmetry C18 3.9 mm x 150 mm
    b) Zorbax Rx 3.0 mm x 150 mm
  - Mobile Phase: 50 mM potassium phosphate, pH 3.0/acetonitrile 55:45
  - Flow Rates: a) 1.0 mL/min
    b) 1.4 mL/min
  - Detector: 240 nm
  - Sample: 600 pg/mL, 10 µL injection

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**Impact of Column Length on Resolution (Approach 1)**

- Symmetry® C, 5 µm, 2.1 x 20 mm
  - Gradient time: 1 minute
- Symmetry® C, 5 µm, 2.1 x 30 mm
  - Gradient time: 1 minute
- Symmetry® C, 5 µm, 2.1 x 50 mm
  - Gradient time: 1 minute

**Conditions:**
- Mobile phase: Ac0.1% TFA/0.1% Ammonium acetate
- Gradient: 0-60% B in noted time
- Column temperature: 30°C
- Detector: 254 nm
- Injection volume: 1 µL
- Flowrate: 1 mL/min

- Maintain resolution when scaling gradient volume proportionally to column volume.
- Reduce analysis time by >50%.

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**Chromatographic System**

- **Pump**
- **Solvent proportioning**
- **Detector**
- **Column Performance & Geometry**
  - Stationary phases' chemistry
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  - 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

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Improvement in Peak Shape for Bases

Conventional C$_{18}$ vs. Modern C$_{18}$

1970's

Modern C$_{18}$

Hybride Silica Technology -- Today

Time (min)

© Waters Corporation

Peak Forms of Complexing Agent (Hinokitiol)

Mobile Phase: 50% 20 mM Phosphate Buffer pH 3.6 with 0.05% EDTA, 50% Acetonitrile

Metal-Free

Contaminated Column

© Waters Corporation

Chromatographic System

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## Solvent Effects

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

**Absorbance AU**

**Wavelength nm**
432 Conductivity Detection of Seven Anion Standard

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 HC
Eluent: Borate/Gluconate
Flow rate: 2.0 mL/min
Injection vol.: 100 µL
Detection: Direct Conductivity

Basic HPLC Review

pH Effects on UV Spectra (L-Ascorbic Acid)

432 Conductivity Detection of Seven Anions with Chemical Suppression

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

Chromatographic System

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Integration Errors Caused by Tailing

**Exponential Skim**
Resolves rider or shoulder peaks from a parent peak.

**Tangential Skim**

The Tangential and Exponential Skim events require the use of a drop line within the parent peak. For best results, add only one drop line per peak that is to be skimmed.

**Peak Detection:**

- **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