Relation between Chromatographic Resolution and Limit of Detection in Spectrophotometric HPLC Detection

Karsten G. Kraiczek[1,3] , Gerard P. Rozing[2] and Roland Zengerle[3]

[1]Agilent Technologies, Hewlett-Packard Strasse 8, Waldbronn, Germany

[2]ROZING.COM Consulting, Karlsruhe, Germany.

[3]IMTEK– Department of Microsystems Engineering, University of Freiburg, Freiburg, Germany

Acknowledgement

The authors want to acknowledge **Prof. Klaus-Peter Hupe** (em.), who initiated these considerations and discussed with us in the eighties of last century while at Hewlett-Packard in Waldbronn Germany, which were first presented at HPLC'1985, Edinburgh, Scotland and which has formed the basis of our current work.

Trends of Spectrophotometric Detection in HPLC

Till the end of the last century:

- Reduction of detector noise and improvement of linear dynamic range has been a major thrust
- Maintaining separation efficiency and peak shape was a prevailing user requirement
- Detector optics and flow cell design have been continuously improved to minimize the influence of adverse physical phenomena like RI sensitivity and temperature fluctuations

Consolidation of flow cell volume and geometry;
cylindrical, 5 – 15 µL, 10 mm path length

Recent Trends in (U)HPLC

- Low flow rate LC (µL-nL/min) in particular for sample limited applications
- Usage of sub-2-µm totally porous or superficially porous particles in short, narrow bore (2.1 mm i.d.) columns → UHPLC

Peak volumes are significantly reduced (10-100x)

Design & Engineering Challenge……

Develop spectrophotometric detectors for (U)HPLC with very low volume flow cells maintaining detection limit, linear dynamic range minimizing RI and temperature effects whilst preserving chromatographic separation integrity.

How does the Flow Cell Design in Spectrophotometric Detectors Affects the (U)HPLC Separation?

Lambert-Beer Law:

$$A_{i,\lambda} = \varepsilon_{i,\lambda} \cdot c_i \cdot L_{cell}$$

$A_{i,\lambda}$ = measured absorbance of solute *i* at wavelength λ
$\varepsilon_{i,\lambda}$ = molar absorption coefficient of solute *i*
c_i = concentration of the solute *i*
L_{cell} = optical/sample path length of detector flow cell

For a solute eluting from the column with a Gaussian peak shape:

$$c_{i,\max} = \frac{Q_{inj,i}}{\sqrt{2\pi} \cdot \sigma_{v_{i,col}}}$$

$c_{i,max}$ = concentration in peak maximum
$Q_{inj,i}$ = amount of sample injected of solute *i*
$\sigma_{v,i}$ = standard deviation of peak profile of solute *i*

In practice however:

$$\sigma^2_{v_{i,tot}} = \sigma^2_{v_{i,col}} + \sigma^2_{v_{inj}} + \sigma^2_{v_{cap}} + \sigma^2_{v_{\det}}$$

(U)HPLC – Extra Column Band-Broadening

$$\sigma^2_{v,tot} = \sigma^2_{v,col} + \sigma^2_{v,cap} + \sigma^2_{v,inj} + \sigma^2_{v,cell}$$

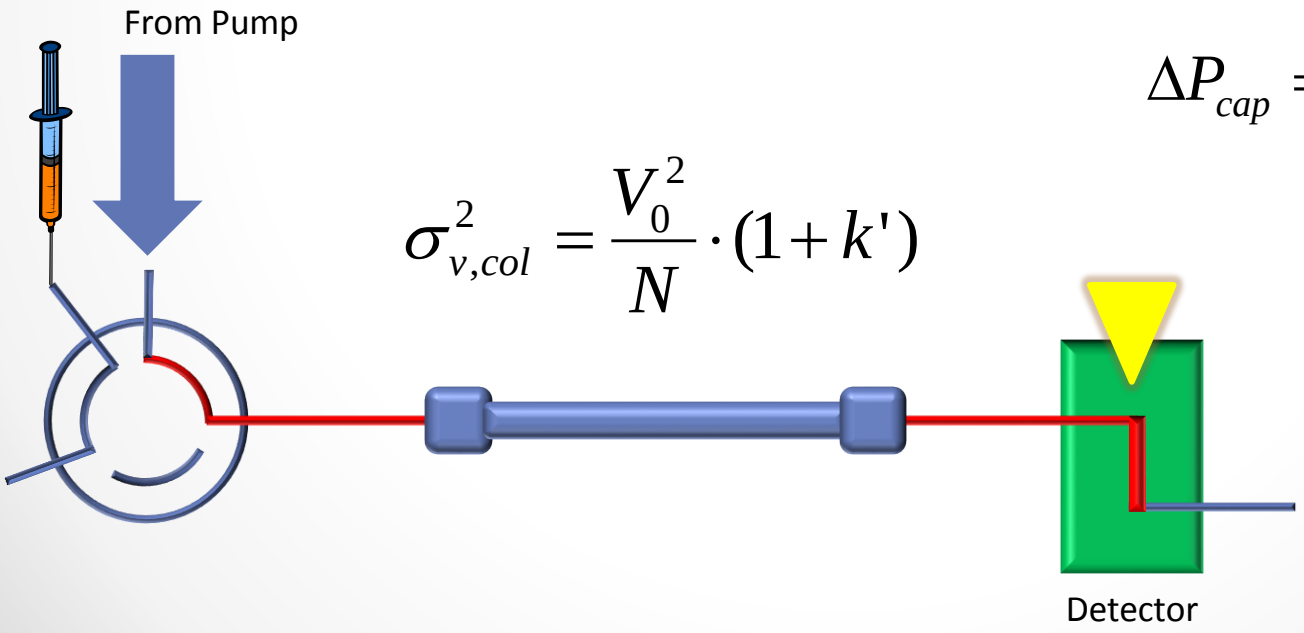
$$\sigma^2_{V,inj} = \frac{V^2_{inj}}{12}$$ (without dispersion)

$$\sigma^2_{V,cap} = \frac{V^2_{cap}}{L} \cdot \frac{r^2 u}{24 D_m}$$ (Golay equation)

Typical values for variance :

Injector	**1 µl²**
Capillaries (500x0.12 mm)	**7 µl²**
Capillaries (500x0.08 mm)	**4 µl²**
Column (2.1x50 mm, k' =1)	**3 µl²**
Column (2.1x50 mm, k' =5)	**33 µl²**

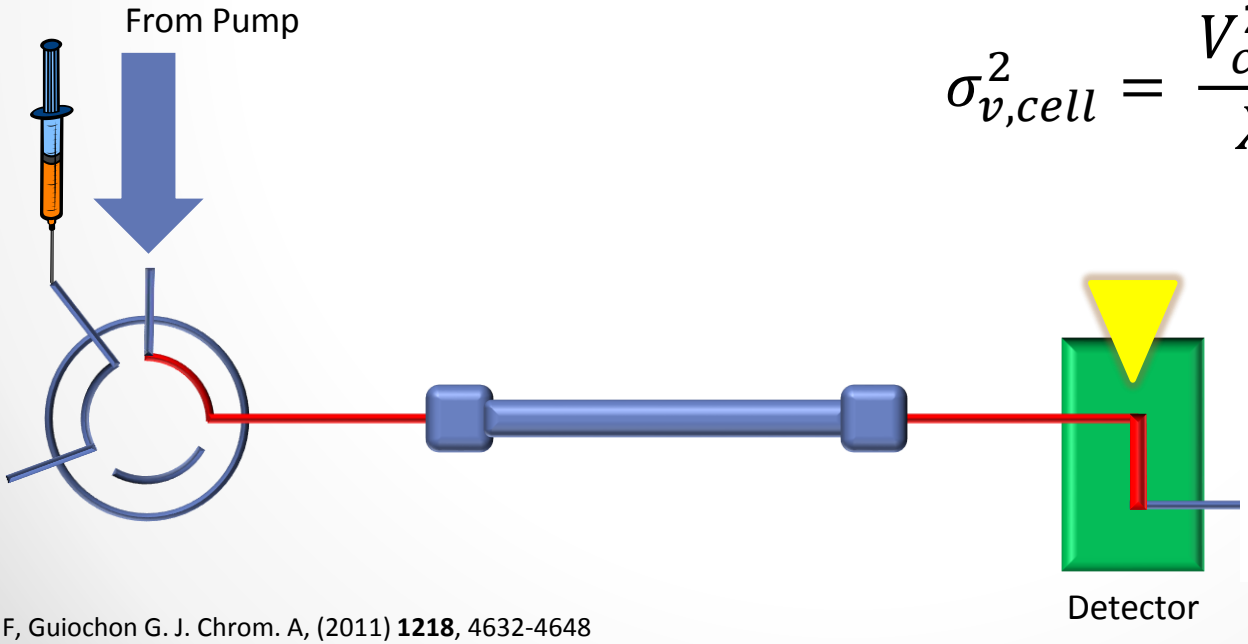
$$\Delta P_{cap} = \frac{F \cdot 8\eta \cdot L_{cap}}{\pi \cdot r^4_{cap}}$$



$$\sigma^2_{v,col} = \frac{V_0^2}{N} \cdot (1 + k')$$

(U)HPLC – Extra Column Band-Broadening

$$\sigma^2_{v,tot} = \sigma^2_{v,col} + \sigma^2_{v,cap} + \sigma^2_{v,inj} + \sigma^2_{v,cell}$$



$$\sigma^2_{v,cell} = \frac{V^2_{cell}}{X}$$

X = dispersion factor

12	(non-dispersive flow cell)
1	(flow cell as ideal mixer)
5-6	(typical value)*

*Gritti F, Guiochon G. J. Chrom. A, (2011) **1218**, 4632-4648

How does the Flow Cell Design in Spectrophotometric Detectors Affects the (U)HPLC Separation?

In practice however:

$$\sigma^2_{v_{i,tot}} = \sigma^2_{v_{inj}} + \sigma^2_{v_{i,col}} + \sigma^2_{v_{cap}} + \sigma^2_{v_{det}}$$

Equation describes well designed HPLC system, where column and flow cell are the dominant sources for dispersion.

minimize

$$\sigma^2_{v_{i,tot}} \cong \sigma^2_{v_{i,col}} + \sigma^2_{v_{det}}$$

$$\sigma^2_{v_{i,tot}} \cong \sigma^2_{v_{i,col}} + \frac{V^2_{cell}}{X}$$

$$c_{i,\max} \cong \frac{Q_{inj,i}}{\sqrt{2\pi} \cdot \sqrt{\sigma^2_{v_{i,col}} + \frac{V^2_{cell}}{X}}}$$

Signal-to-Noise Ratio in Spectrophotometric Detection

Extending Baumann's Principle*,*,*

Lambert Beer's law

$$A_{i,\lambda} = \varepsilon_{i,\lambda} \cdot c_{i,\max} \cdot L_{cell} = \log\left(\frac{m_{0,\lambda}}{m_{\lambda}}\right)$$

Photon flux converted into electrical signal

$$m_{0,\lambda} = M_{\lambda} \cdot \Delta\lambda \cdot G \cdot T_{optic,\lambda} \cdot \eta_{\lambda}$$

Optical Signal ~ chromatographic Signal

$$S = \left(m_{0,\lambda} - m_{\lambda}\right) \cdot \tau$$

Standard deviation of photon flux = rms Noise

$$N_{rms} = \sqrt{m_{0,\lambda} \cdot \tau}$$

The rms-value of the Signal-to-Noise Ratio

$$SNR_{rms} = \frac{\left(m_{0,\lambda} - m_{\lambda}\right) \cdot \tau}{\sqrt{m_{0,\lambda} \cdot \tau}}$$

$$LOD \propto \frac{1}{SNR}$$

M_{λ} = spectral output of light source
$\Delta\lambda$ = spectral bandwidth
G = smallest light conductivity of optical system
$T_{optic,\lambda}$ = overall transmission of optic
η_{λ} = quantum efficiency of photo detector at λ
τ = detectors time constant
$m_{0,\lambda}$ = fraction of photon flux converted into electrons
m_{λ} = reduced photon flux caused by sample absorption
$\varepsilon_{i,\lambda}$ = molar extinction coefficient of solute i
c_i = concentration of the solute i
L_{cell} = optical, sample path length of detector flow cell

1st order of Taylor expansion Lambert-Beer's law

$$\frac{m_{0,\lambda} - m_{\lambda}}{m_{0,\lambda}} = 2.3 \cdot \varepsilon_{i,\lambda} \cdot c_i \cdot L_{cell}$$

$$SNR_{rms} = 2.3 \cdot \varepsilon_{i,\lambda} \cdot c_{i,\max} \cdot L_{cell} \cdot \sqrt{M_{\lambda} \cdot \Delta\lambda \cdot G \cdot T_{optic,\lambda} \cdot \eta_{\lambda} \cdot \tau}$$

*W. Baumann, Z. Anal. Chem. 284, 31-38 (1977)

*,*K. Kraiczek et al., Anal. Chem., 2013, 85 (10), pp 4829–4835

The Dilemma in Spectrophotometric HPLC detection*,*

*in HPLC detection the ptp value of SNR is relevant

Signal Parameters *Optical Parameters, Detector Settings*

$$SNR_{ptp}(L_{cell}, V_{cell}) = \frac{2.3}{6} \cdot \varepsilon_{i,\lambda} \cdot c_{\max,i} \cdot L_{cell} \cdot \sqrt{M_\lambda \cdot \Delta\lambda \cdot G \cdot T_{optics,\lambda} \cdot \eta_\lambda \cdot \tau}$$

$$c_{i,\max} \cong \frac{Q_{inj,i}}{\sqrt{2\pi} \cdot \sqrt{\sigma^2_{v_{i,col}} + \frac{V^2_{cell}}{X}}}$$

G_{optics} or $G_{conv-cell} = f(L_{cell}, V_{cell})$

- To obtain best chromatographic resolution $\Rightarrow V_{cell} \to 0$ *but* $\Rightarrow SNR \to 0$
- Not always obvious on first glance but both extremes for the cell volume and path length lead to:

$$V_{cell} \to 0 \ \wedge \ V_{cell} \to \infty \Rightarrow SNR \to 0$$
$$L_{cell} \to 0 \ \wedge \ L_{cell} \to \infty \Rightarrow SNR \to 0$$

- Hence, there must be an optimal flow cell volume and path length for ***best SNR*** for each chromatographic condition. *Question:* $V_{cell,opt_{SNR}} = ?$ $L_{cell,opt_{SNR}} = ?$

=> Compromise to be made between $R_s \vee SNR$?

Quantitative Model ?
Close theoretical gap !

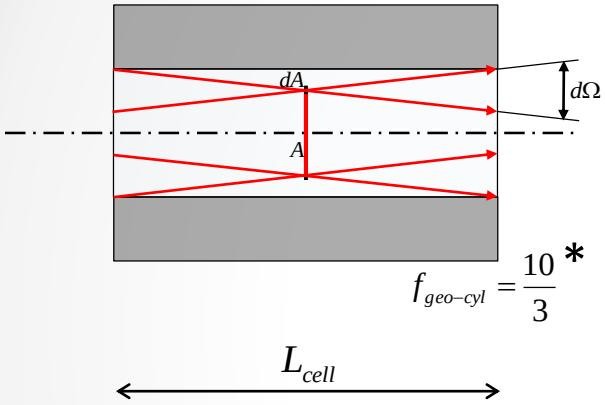
*,*K. Kraiczek et al., Anal. Chem., 2013, 85 (10), pp 4829–4835

Light Conductivity (Light Throughput)

Conventional and Liquid Core Waveguide Flow Cells

$$G = \int_A \int_\Omega dA \cdot d\Omega$$

e.g. Conventional Cylindrical Flow Cell

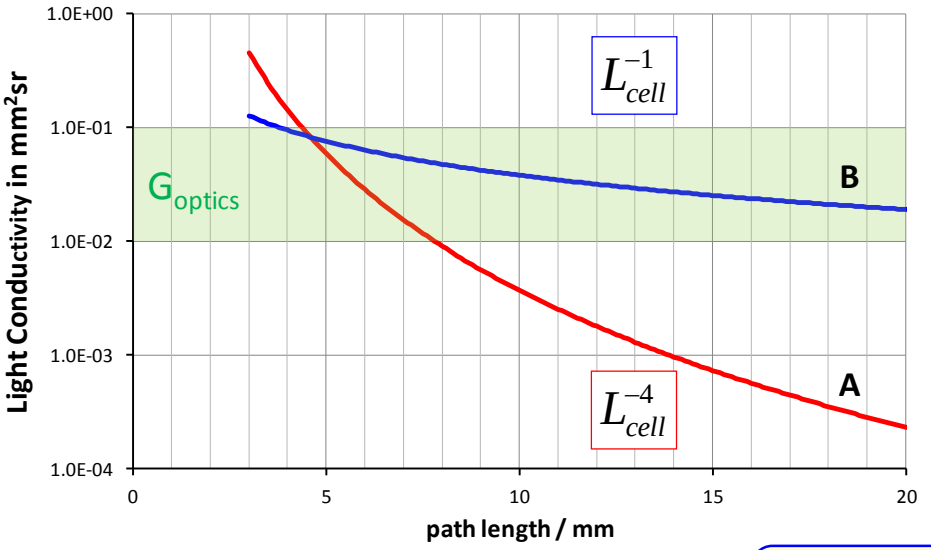


$$f_{geo-cyl} = \frac{10}{3}\ ^*$$

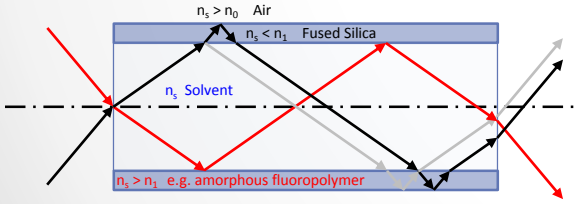
$$G_{conv-cell} \cong f_{geo} \cdot n_{solv}^2 \cdot \frac{V_{cell}^2}{L_{cell}^4} \cdot \Omega_0$$

Typically large in volume and short in path length

e.g. flow cell volume 2.5 µL



LCW Flow Cell



$$G_{LCW-cell} = \pi \cdot NA_{cell}^2 \cdot A_{cell} \cdot \Omega_0$$

$$G_{LCW-cell} = \pi \cdot NA_{cell}^2 \cdot \frac{V_{cell}}{L_{cell}} \cdot \Omega_0$$

Allow longer path length even at lower volume

$$NA_{cell} = \sqrt{n_{solv}^2 - n_{clad}^2}$$

*W. Baumann, Z. Anal. Chem. 284, 31-38 (1977)

Optimizing Signal-to-Noise Ratio

A. Conventional Cylindrical Flow Cell

Signal Parameters *Optical Parameters, Detector Settings*

$$SNR_{ptp}(L_{cell}, V_{cell}) = \frac{2.3}{6} \cdot \varepsilon_{i,\lambda} \cdot c_{\max,i} \cdot L_{cell} \cdot \sqrt{M_\lambda \cdot \Delta\lambda \cdot G \cdot T_{optics,\lambda} \cdot \eta_\lambda \cdot \tau}$$

$$c_{i,\max} \cong \frac{Q_{inj,i}}{\sqrt{2\pi} \cdot \sqrt{\sigma^2_{v_{i,col}} + \frac{V^2_{cell}}{X}}}$$

$$G_{optics} \quad or \quad G_{conv-cell} = f_{geo} \cdot n^2_{solv} \cdot \frac{V^2_{cell}}{L^4_{cell}} \cdot \Omega_0$$

Case 1:

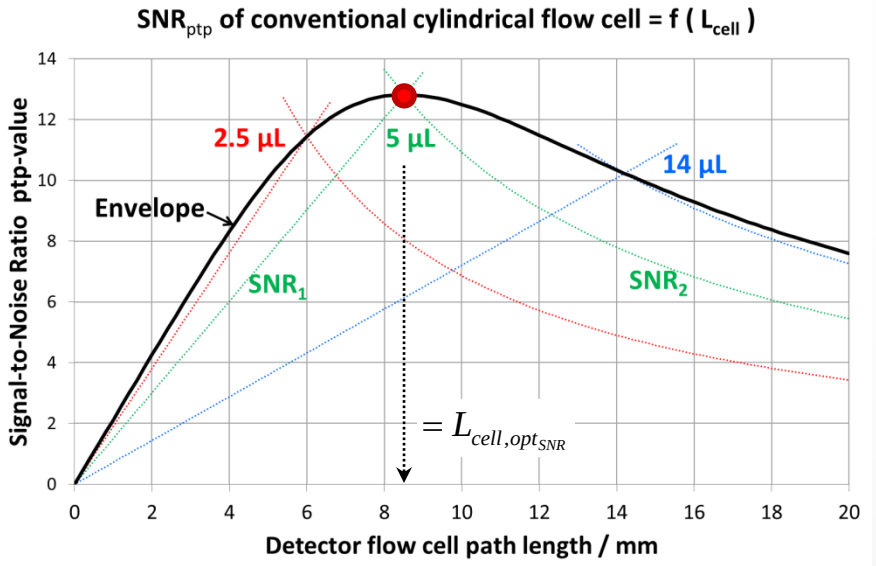
Optics is limiting light throughput

$$SNR \propto L_{cell}$$

Case 2:

Flow Cell is limiting light throughput

$$SNR \propto \frac{1}{L_{cell}}$$



$C_8H_{10}N_4O_2$

$V_{cell,opt} = ???$

Parameters used to illustrate approach:

Optics: λ= 273 nm; M_λ= 1·10^{15} 1/(s sr cm^2 nm); Δλ= 4 nm; G_{optics}= 0.03 mm^{2}sr; T_{optics}= 0.14; η_λ= 0.25; τ= 1 s.
Sample: Caffeine in Water; c_0= 30 pg/µL; V_{inj}= 1.0 µL; Q_{inj}= 154.5 fmol; ε_λ= 9900 Liter/(mol cm).
Chromatography: σ^2_{col}= 5 µL^2 {e.g. d_{col}= 2.1 mm; L_{col}= 50 mm; d_p= 1.8 µm; N= 10000; ε_{tot}= 0.52; k= 1.5}. X= 5.

Optimizing Signal-to-Noise Ratio

A. Conventional Cylindrical Flow Cell SNR = f (V_{cell})

SNR = f (flow cell volume)

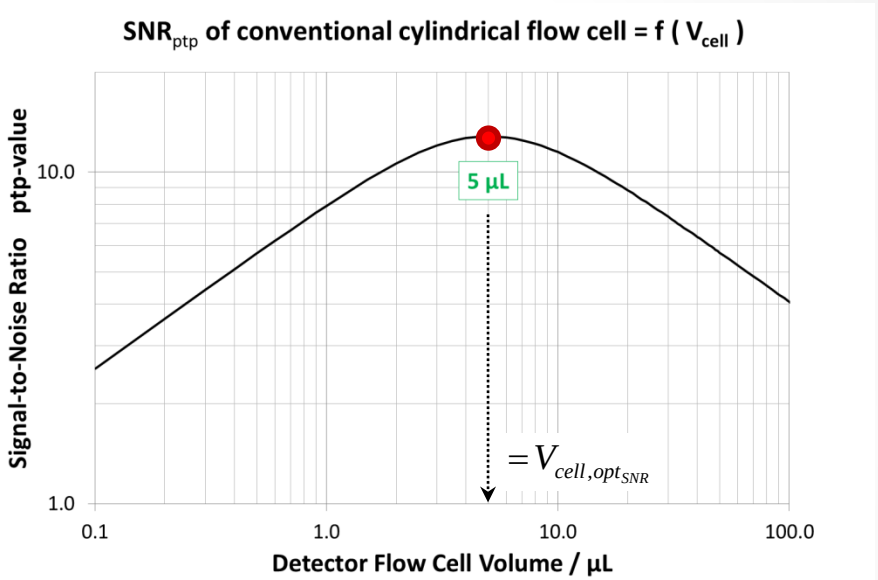
$$SNR(V_{cell}) = f_3 \cdot \sqrt{\frac{V_{cell}}{\sigma^2_{v_{i,col}} + \frac{V^2_{cell}}{X}}}$$

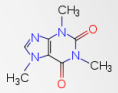
SNR curve has a clear optimum

$$V_{cell,opt\ SNR} = \sqrt{X} \cdot \sigma_{v_{i,col}}$$

'Volume Variance' Matching for best SNR

$$\sigma^2_{v,cell} = \sigma^2_{v_i,col}$$





$C_8H_{10}N_4O_2$

Parameters used to illustrate approach:
Optics: λ= 273 nm; M_λ= 1·10^{15} 1/(s sr cm² nm); Δλ= 4 nm; G_{optics}= 0.03 mm²sr; T_{optics}= 0.14; η_λ= 0.25; τ= 1 s.
Sample: Caffeine in Water; c_0= 30 pg/µL; V_{inj}= 1.0 µL; Q_{inj}= 154.5 fmol; ε_λ= 9900 Liter/(mol cm).
Chromatography: σ^2_{col}= 5 µL^2 {e.g. d_{col}= 2.1 mm; L_{col}= 50 mm; d_p= 1.8 µm; N= 10000; ε_{tot}= 0.52; k= 1.5}. X= 5.

- Match Volume Variances of Flow Cell and Eluting Compound for ***best SNR***.
- *!! Not necessarily best conditions for chromatography but an important step toward quantitative model !!*

Scaling in X and Y

$$Z = \frac{\sigma^2_{v,cell}}{\sigma^2_{v,col}} \qquad Z \in R_0^+$$

$$SNR_{NORM} = \sqrt{\frac{2 \cdot \sqrt{Z}}{1+Z}} \qquad SNR_{NORM} \in [0;1]$$

Optimizing Signal-to-Noise Ratio

B. Liquid Core Waveguide Flow Cell

Signal Parameters *Optical Parameters, Detector Settings*

$$SNR_{ptp}(L_{cell},V_{cell}) = \frac{2.3}{6}\cdot\varepsilon_{i,\lambda}\cdot c_{\max,i}\cdot L_{cell}\cdot\sqrt{M_\lambda\cdot\Delta\lambda\cdot G\cdot T_{optics,\lambda}\cdot\eta_\lambda\cdot\tau}$$

$$c_{i,\max} \cong \frac{Q_{inj,i}}{\sqrt{2\pi}\cdot\sqrt{\sigma^2_{v_{i,col}}+\frac{V^2_{cell}}{X}}}$$

$$G_{optics} \quad or \quad G_{TIR-cell} = \pi\cdot NA^2\cdot\frac{V_{cell}}{L_{cell}}\cdot\Omega_0$$

Case 1:

Optics is limiting light throughput

$$SNR \propto L_{cell}$$

Case 2:

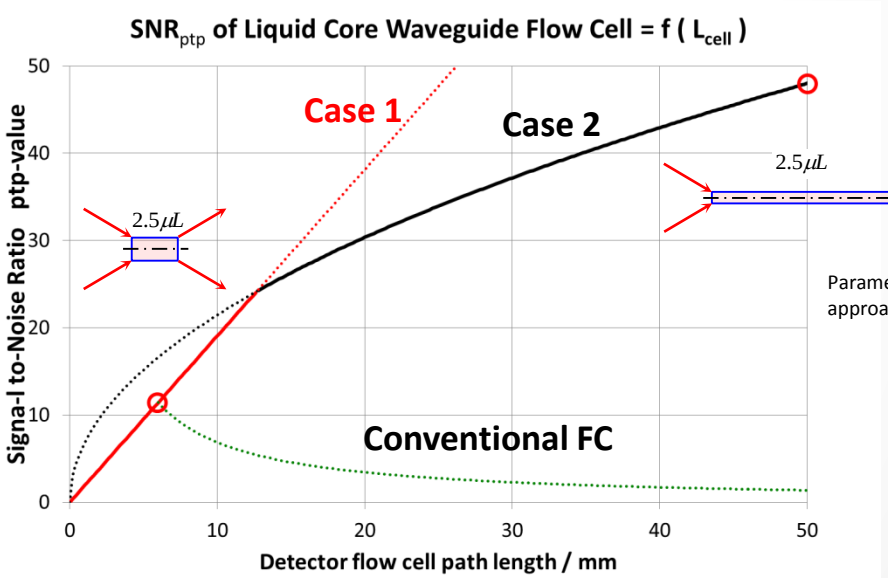
Flow Cell is limiting light throughput

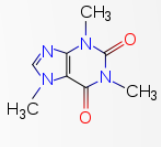
$$SNR \propto \sqrt{L_{cell}}$$

$$SNR_{TIR}(L_{cell},V_{cell}) = f_{TIR}\cdot\sqrt{\frac{V_{cell}}{\sigma^2_{v_{i,col}}+\frac{V^2_{cell}}{X}}}\cdot\sqrt{L_{cell}}$$

$$V_{cell,opt\,SNR} = \sqrt{X}\cdot\sigma_{v_{i,col}}$$

$$L_{cell,opt} = ?$$





Parameters used to illustrate approach same as previous slide.

*Make LCW flow cells rather **long** than short for best **SNR.***

*Reasons to limit path length are **excessive solvent absorption** and desired **LDR.***

SNR and Chromatographic Resolution

How is SNR coupled to Resolution?

$$R_s = \frac{t_{R_2} - t_{R_1}}{\left(\frac{w_{b_2} + w_{b_1}}{2}\right)} = \frac{\Delta t_R}{w_b} = \frac{\Delta t_R}{4 \cdot \sigma_t}$$

❑ Definition

$$0 < R_{s,tot} < R_{s,col}$$

❑ Resolution Domain

$$R_{loss} = \frac{R_{s,tot} - R_{s,col}}{R_{s,col}} \qquad R_{loss} \in [-1; 0]$$

❑ Definition Relative Loss in Resolution

$$Z = \frac{\sigma^2_{t,cell}}{\sigma^2_{t,col}} = \frac{\sigma^2_{v,cell}}{\sigma^2_{v,col}} \qquad Z \in R_0^+$$

❑ Variance Ratio

$$R_{loss} = \frac{1}{\sqrt{Z+1}} - 1$$

❑ Resolution Loss = f (Variance Ratio)

$$SNR_{NORM} = \sqrt{\frac{2 \cdot \sqrt{Z}}{1+Z}} \qquad SNR_{NORM} \in [0; 1]$$

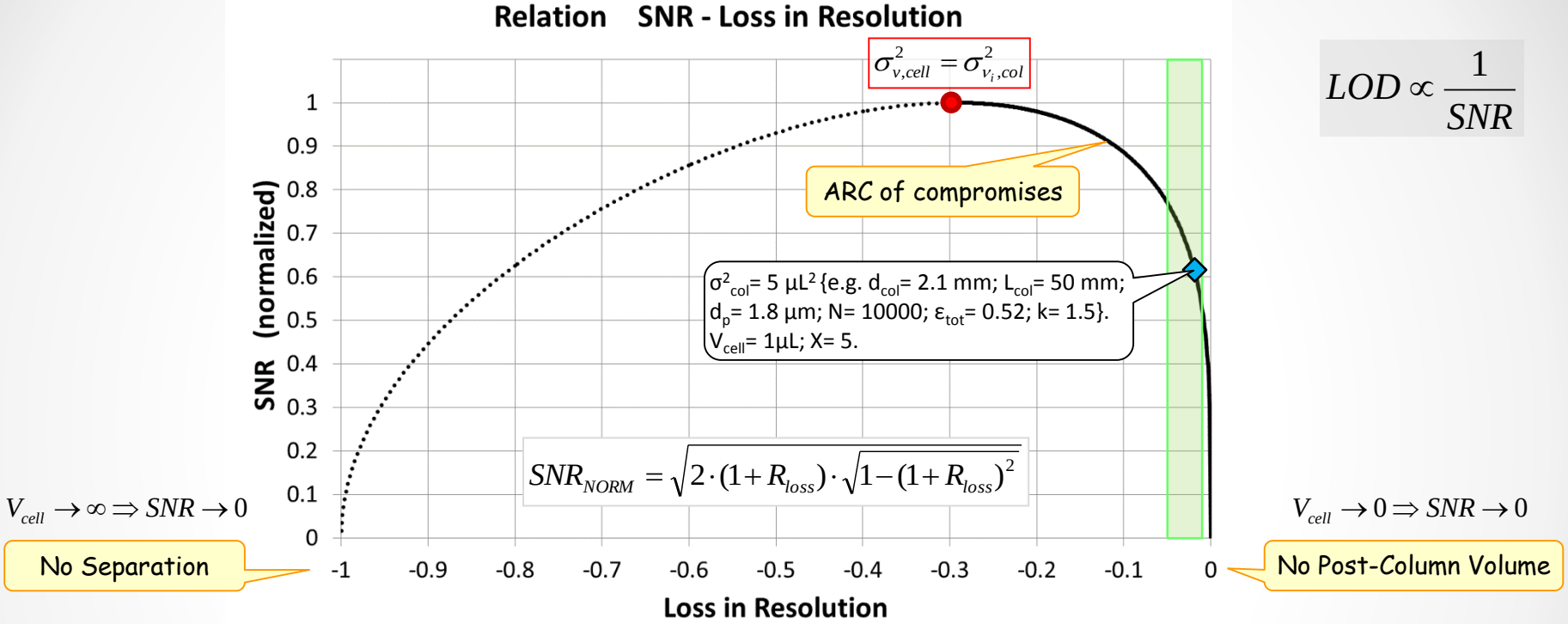
❑ SNR = f (Variance Ratio)

$$SNR_{NORM} = \sqrt{2 \cdot (1+R_{loss}) \cdot \sqrt{1-(1+R_{loss})^2}}$$

❑ SNR = f (Resolution Loss)

SNR and Chromatographic Resolution

Compromises: *Uncertainty Principle* in Spectrophotometric HPLC Detection



$$LOD \propto \frac{1}{SNR}$$

- Optimization strategy for best SNR by Matching Volume Variance of column and flow cell (Z=1) is resulting in a loss of -30% in peak resolution.
- Less than -10%: SNR deteriorates relatively quickly with small gains only in resolution
- Small operational range for good flow cell designs.
- The actual "System Operating Point" depends on: retention factor *k*, column and flow cell optical parameters and the flow cells dispersion factor X.

Conclusions

- New Quantitative Model describes the trade-off between Chromatographic Resolution and SNR.
- Model recommends and confirms that detector flow cell should be dimensioned according to resolution requirements rather than to best SNR.
- Model suggests following equation for the max flow cell volume:

$$V_{cell} = \sigma_{v_{i,col}} \cdot \sqrt{X \cdot \left(\frac{1}{(1+r_{loss})^2} - 1 \right)}$$

Specify your resolution requirements.

  - Improved dispersion behavior allows larger flow cell volume.
- Liquid Core Waveguide flow cells have, by principle, better SNR performance over conventional flow cells.
  - Reasons to limit their path length are excessive solvent absorption and desired LDR.
- Adaptive flow cells would be ideal but probably will remain a dream

Reprints

A PDF copy of this talk will be available on my website
http://www.rozing.com after the talk (registration required)