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Relation between Chromatographic Resolution and Signal-to-Noise Ratio in Spectrophotometric HPLC Detection

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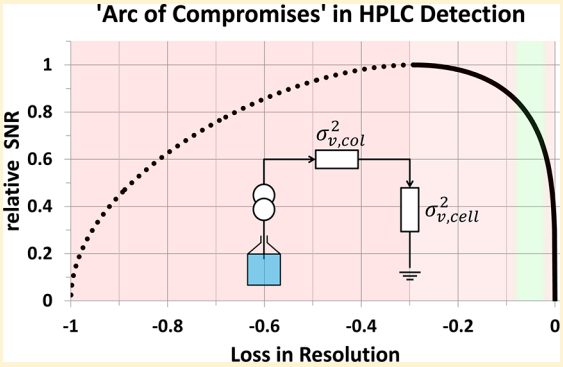
ABSTRACT: Absorption spectrophotometry has been and still is the industry standard for detection in HPLC. Limit of detection (LOD) and linear dynamic range (LDR) are the primary performance requirements and have driven continuous improvement of spectrophotometric HPLC detectors. Recent advances in HPLC column technology have led to low flow-rate HPLC such as capillary HPLC and nanoflow HPLC and put higher demands on optical HPLC signal detection. However, fundamental principles in spectrophotometric HPLC detection have not been reviewed for many years. In particular the relationship between the detector's signal-to-noise ratio (SNR) and band broadening needs to be re-evaluated. In this work, a new quantitative model is presented which allows the calculation of the trade-off made between chromatographic resolution and SNR in spectrophotometric HPLC detection. Modern optics flow cells based on total internal reflection are included and compared to conventional flow cells.



In the late 1960s, pioneers in liquid chromatography like Giddings,[1] Huber,[2] Guiochon,[3] and Knox[4] described how to select column dimensions, stationary phase properties and conditions for optimal operation of HPLC columns with respect to analysis time, separation efficiency, and detection limit. At the time when these treatments were made, the operating range of HPLC systems was hypothetical. Column and instrumentation technology have since evolved, allowing reliable and user-friendly operation of HPLC systems in which typically 4.6 mm i.d. columns with 5−10 μm particles were used and operated at 1−5 mL/min flow rate, with the separation temperature between ambient and 80 °C and injection volumes ranging from 5 to 25 μL. The volume of detection flow cells were typically in the order of 10 μL with an optical path length of 10 mm. Maximal operating pressure was 400 bar. Such systems have become the standard and the workhorse of analytical chemists.

Besides these standard systems in the late eighties and early nineties, HPLC systems using low i.d. columns (<1 mm) became established, particularly in cases where the sample amount was limited.[5,6] Drug metabolism studies and pharmacokinetics for new drug development,[7] and, in particular, the analysis of high molecular weight biomolecules in life science research[8] have been drivers for this development. Commercial HPLC systems for capillary[9] and nanoflow[10] HPLC appeared on the market with MS and spectrophotometric detection. Although in most instances mass spectrometry is used for detection, UV−Vis spectrophotometric detection is used in routine work leading to compromised peak fidelity and detection limit since the system and detection volumes are too large relative to the sample volume in many cases.

The development in HPLC column technology has accelerated in the past decade. Very small particles with diameters less than 2 μm (sub-two-micrometer, STM) became popular as a pathway to ultrahigh separation efficiency per unit of time or per unit length. Most recently, HPLC columns with so-called "porous shell core" particles, having a 0.5 μm porous outer shell on a 1−2 μm impervious core, have been introduced.[11] At the same time, column diameters and lengths were reduced (e.g., i.d. 1−2 mm, 50 mm). From such columns, the solutes elute in very small zones of high concentration offering the potential to achieve a better signal-to-noise ratio (SNR).

These advances in HPLC column technology are re-emphasizing a dilemma in spectrophotometric detection from the early days of HPLC system technology. Lambert−Beer's law mandates that the path length of the flow cell be long in order to obtain a high absorbance signal. On the other hand, the detector flow cell should be of low volume to prevent zone dispersion, resulting in lower light throughput and increased noise. With these constraints in mind, volume and light throughput of detector flow cells are optimized by making a compromise between zone dispersion and best SNR. The objective of this work is to bridge the theoretical gap between chromatographic resolution and SNR. Modern optics flow cell concepts based on total internal reflection (TIR)[12,13] will be included in this work and compared to conventional flow cells.

THEORY

Sample Dispersion. Spectrophotometric detection is governed by Lambert−Beer's law:

$$A_{i,\lambda} = \varepsilon_{i,\lambda} c_i L \tag{1}$$

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$A_{i,\lambda}$ is the measured absorbance of solute i at wavelength λ, $\varepsilon_{i,\lambda}$ the molar absorptivity, c_i the concentration, and L the path length of absorption. In a chromatographic separation, the concentration response function of the system $c(V,t)$ is the convolution product of several zone dispersion processes and assumed to be of Gaussian shape (Figure 1). The height of a chromatographic peak is given by

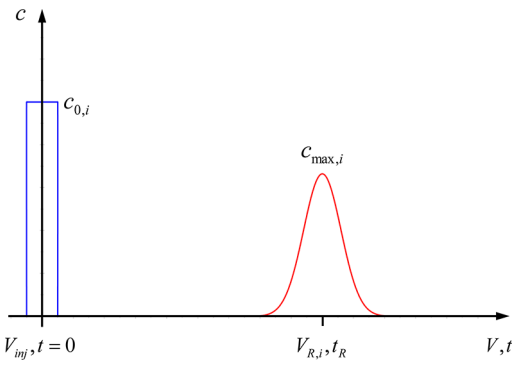

Figure 1. Zone dispersion in a chromatographic system; $c_{0,i}$ = sample concentration of solute i at injection; $c_{max,i}$ = max peak concentration of solute i in detector flow cell; V_{inj} = injection volume; $V_{R,i}$ = retention volume of solute i; t_R = retention time.

$$c_{max,i} = \frac{Q_{inj,i}}{\sqrt{2\pi}\,\sigma_{\nu_i,tot}} \tag{2}$$

$Q_{inj,i}$ is the amount of sample injected of solute i and $\sigma_{\nu_i,tot}$ is the total standard deviation of a solute's zone dispersion in the chromatographic system in volume units. The total standard deviation can be calculated using the addition of variances,[14] where the total variance of the measured peak is equal to the sum of the column peak variances plus the variances from the injector, the connection tubing, and the detector flow cell.

$$\sigma_{\nu_i,tot}^2 = \sigma_{\nu_i,col}^2 + \sigma_{\nu,inj}^2 + \sigma_{\nu,cap}^2 + \sigma_{\nu,cell}^2 \tag{3}$$

All individual variance contributions can be experimentally determined relatively well[15,16] but are not always easy to predict in theory.[15,16] Generally it is desirable to keep the variance contributions from the injector and all connection tubing (from the point of injection to the detector flow cell) as small as possible compared to the separation column and the detector flow cell. This is typically true for standard HPLC systems with a column i.d. of 2.1−4.6 mm but difficult to achieve using traditional systems with narrow columns (≤1.0 mm i.d.). In the search for the theoretical link between chromatographic resolution and SNR it is convenient to reduce eq 3 to 4, which describes a well-designed HPLC system where the separation column and the detector flow cell are the dominant sources for dispersion.

$$\sigma_{\nu_i,tot}^2 \cong \sigma_{\nu_i,col}^2 + \sigma_{\nu,cell}^2 \tag{4}$$

The contribution of the volume of the detector flow cell to peak dispersion can be generally described as in eq 5.

$$\sigma_{\nu,cell}^2 = \frac{V_{cell}^2}{X} \tag{5}$$

with

$$X \in [1;\ 12]$$

V_{cell} is the volume of the detector flow cell and X is a factor that has been introduced to describe the dispersion behavior of the detector flow cell, e.g., X = 1 if the flow cell is an ideal mixer[17] or X = 12 for a nondispersive cell.[17] Both extreme values for X are, of course, not good representations. Detector flow cells can be considered to a first approximation as very short cylindrical tubes. Atwood and Golay[18] estimated a dispersion factor of X = 3 from the parabolic profile in such tubes assuming no diffusion of the analyte. However, values of 5−6 for X are generally closer to reality.[16] Substitution of eq 5 in eq 4 and the resulting equation in eq 2 yields eq 6, the chromatographic peak height expressed in concentration units:

$$c_{max,i} \cong \frac{Q_{inj,i}}{\sqrt{2\pi}\sqrt{\sigma_{\nu_i,col}^2 + \frac{V_{cell}^2}{X}}} \tag{6}$$

Equation 6 is the theoretical recommendation to miniaturize chromatographic systems in order to obtain high concentration signals. It mandates the use of low volume, short and in particular narrow separation columns with high plate numbers, detector flow cells of low volume and low dispersion, and to minimize or better eliminate connecting volumes. Strictly speaking, eq 6 holds under the assumption that there is no overloading of the column or the flow cell.

Signal-to-Noise Ratio. The principle of Baumann[19] will be extended in the following to derive an SNR expression that includes not only optical but also all relevant chromatographic parameters.

The fraction of photon flux $m_{0,\lambda}$ (photons per unit time) at wavelength λ that is converted into a useable electrical signal by the photo detector is given by

$$m_{0,\lambda} = M_\lambda \Delta\lambda G T_{optics,\lambda} \eta_\lambda \tag{7}$$

M_λ is the spectral photon flux density of the detector light source, $\Delta\lambda$ the detector's spectral bandwidth, G the light conductivity of the optical system, $T_{optics,\lambda}$ the overall transmittance of the optics, and η_λ the quantum efficiency of the photo detector at wavelength λ. One of the major challenges in spectrophotometric detection is to measure a small difference between two relatively large intensity signals:

$$S = (m_{0,\lambda} - m_\lambda)\Delta t \cong (m_{0,\lambda} - m_\lambda)\tau \tag{8}$$

where S is the optical signal which is proportional to the chromatographic signal, m_λ is the reduced photon flux caused by sample absorption, and τ is the detector's response time. The root-mean-square (rms) value of the noise is the standard deviation of the photon flux:

$$N_{rms} = \sqrt{m_{0,\lambda}\tau} \tag{9a}$$

$$N_{ptp} \cong 6N_{rms} \tag{9b}$$

The peak-to-peak (ptp) value of the noise (eq 9b) is normally used in HPLC detection to describe the detector's noise performance.[20] It is approximately equal to 6 times the rms value. The SNR (ptp) can be written as

$$SNR_{ptp} = \frac{(m_{0,\lambda} - m_\lambda)\tau}{6\sqrt{m_{0,\lambda}\tau}} \tag{10}$$

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Concerning the lower limit of detection, the SNR near zero absorbance is of interest and the approximated form of Lambert–Beer's law, the first order of Taylor expansion of eq 11a, is used to express small $\Delta m_\lambda/m_\lambda$ values:

$$m_\lambda = m_{0,\lambda}\, e^{-2.3\varepsilon_{i,\lambda} c_{\max,i} L_{\text{cell}}} \tag{11a}$$

$$\frac{\Delta m_\lambda}{m_\lambda} \cong \frac{m_{0,\lambda} - m_\lambda}{m_{0,\lambda}} = 2.3\varepsilon_{i,\lambda} c_{\max,i} L_{\text{cell}} \tag{11b}$$

Combining eqs 7 and 10 and 11b yields the equation for the signal-to-noise ratio for a shot noise limited detector:

$$\text{SNR}_{\text{ptp}} = \frac{2.3}{6}\varepsilon_{i,\lambda} c_{\max,i} L_{\text{cell}} \sqrt{M_\lambda \Delta\lambda G T_{\text{optics},\lambda} \eta_\lambda \tau} \tag{12}$$

The signal parameters are represented by Lambert–Beer's law. The square root of the noise parameters mainly reflects the photon flux through the optical system. The flow cell path length and its volume are hidden parameters in the maximum concentration of the chromatographic peak $c_{\max,i}$ and in the light conductivity G.

An optical detector can be considered to be shot-noise limited when all other noise contributions are small in comparison. However, a shot-noise limited detector is not necessarily a good detector. It depends on the amount of light available. As of today deuterium (D_2) discharge lamps are the only reasonable suitable light sources for absorption detectors. These are relatively stable but have weak light output in the UV-range. Details on absolute spectro-radiometric quantities for D_2 lamps can be found, in example, in the investigation by NIST and PTB.[21] Further, the losses in light throughput along the optical path of today's detector optics with up to 80–90% are significant. The photon flux that is converted into a photocurrent is in the order of 10^{11} to 10^{12} photons per second (eq 7). Better absorbance noise values than, e.g., 10^{-5} to a few 10^{-6} AU_{ptp} at a time constant of 1 s are not possible due to shot-noise limitation. Current absorption detectors come very close to these noise values for several reasons. While D_2 lamps have not really improved in light output over the last 30 years, instrument designers have learned to operate these lamps in a more stable way through better supply electronics and thermal designs. Intensity fluctuations (flicker noise) of the light source are often compensated by optical referencing which significantly helps to extend the shot-noise limited range of the detector. The detector flow cell can be an extremely "lively" optical element, and the refractive index induced noise is very difficult to predict in theory or not predictable at all. Refractive index induced noise is best minimized by good optical and thermal flow cell designs (tapered beam flow cell,[22] light waveguide flow cell[13]) and/or with the help of optical referencing. Concerning the limit of detection, the contribution of the electronic noise to the total noise is negligible.

From a technical perspective it is always desirable to strive for a shot-noise limited detector; the more photons available per time unit the better the SNR becomes. To bridge the theoretical gap between chromatographic resolution and SNR, it is convenient to assume a shot-noise limited detector.

Light Conductivity of Flow Cell and Optics. The optimization of SNR makes it necessary to discuss the light conductivity of detector flow cells in detail. The light conductivity (or light throughput) is a geometrical property of an optical system, which characterizes how "spread out" the light is in area and angle.[23] For an optical system consisting of two cross-sectional areas A_1 (e.g., entrance window) and A_2 (e.g., entrance pupil), separated by distance a, the light conductivity is given by

$$G_{\text{optics}} = \frac{A_1 A_2}{a^2}\Omega_0 \tag{13}$$

In an ideal situation, the light conductivity is constant throughout the optical system. In practice, however, the element with the lowest light conductivity, which in HPLC detection is typically that of the flow cell, determines the overall light conductivity. The values for spectrophotometric HPLC detector optics roughly range between 0.01 and 0.1 mm^2 sr.

Conventional (nonlight waveguide) flow cells are still widely used in HPLC detection. The detection volume is typically of cylindrical or conical shape. Both geometries share the same volume-to-length relation for the light conductivity, if the length-to-diameter ratio is not too low (i.e., >3). A very good approximation for the maximum light conductivity that can be reached is, similar to Baumann,[19] given by

$$G_{\text{conv-cell}} \cong f_{\text{geo}}\, n_{\text{solv},\lambda}^{\,2} \frac{V_{\text{cell}}^{\,2}}{L_{\text{cell}}^{\,4}}\Omega_0 \tag{14}$$

where f_{geo} represents a geometrical form factor, e.g., $^{10}/_{3}$ for a cylindrical flow cell, and $n_{\text{solv},\lambda}$ is the refractive index of the solvent in the flow cell. From eq 14, it follows that a conventional flow cell should be short rather than long, and of large volume, for the highest light throughput. However, at the point where the light conductivity of the optical system is starting to limit the light throughput, the flow cell should not be made any shorter.[19]

Light waveguide flow cells have become very popular in HPLC detection. Optically speaking they are simply apertures with light throughput in first approximation independent of the sample path length. This allows increasing the chromatographic signal without increasing the noise provided that an increase in flow cell volume is acceptable by the chromatography. In some designs, total reflection occurs at the interface between the liquid and the inner wall of the flow cell. To achieve this, an amorphous fluoropolymer is often used as low refractive index wall material.[12,24] Other designs prefer to have the total reflection occur at the outer surface of a chemically inert wall material such as fused silica.[13] The numerical aperture can be as high as 0.9, e.g., when total reflection occurs against air. The light conductivity of a light waveguide or total internal reflection (TIR) flow cell is proportional to the square of its numerical aperture $NA_{\text{TIR-cell}}$ and its cross-sectional area $A_{\text{TIR-cell}}$.

$$G_{\text{TIR-cell}} = \pi \text{NA}_{\text{TIR-cell}}^{\,2} A_{\text{TIR-cell}}\Omega_0 \tag{15}$$

It is important to point out that the numerical aperture of a light waveguide flow cell varies with the refractive index of the solvent ($NA = (n_{solv}^{\,2} - n_{clad}^{\,2})^{1/2}$). This can cause changes in light transmission of up to 10% and can lead to unacceptable baseline deviations in a chromatogram, e.g., in gradient mode. This is especially problematic when the difference in the refractive index between the solvent and the clad is small. To avoid refractive index effects on the numerical aperture and to avoid increased risk of refractive index induced noise, the numerical aperture of light waveguide flow cells is typically limited by other components of the optics, e.g., by the use of optical fibers.

Figure 2 shows how drastically the light conductivity of a conventional cylindrical detector flow cell (A) at given volume is dropping with path length, several orders of magnitude over a relatively short path length range. The nature of a light waveguide flow cell (B) of the same volume is much more

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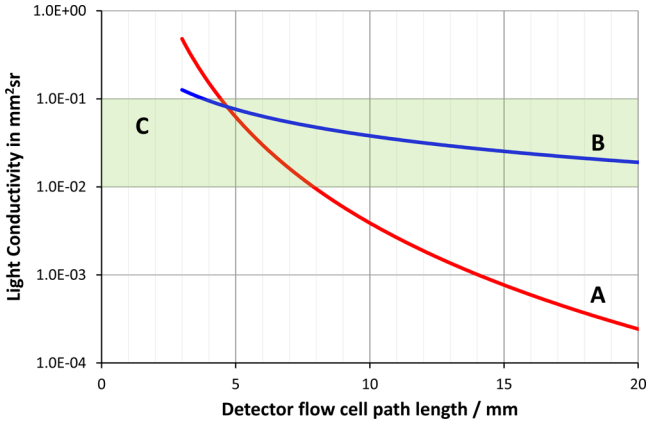

Figure 2. Light conductivity in comparison: (A) Conventional cylindrical flow cell filled with water. Equation 14 with V_{cell} = 2.5 μL; $f_{geo} = {}^{10}/_{3}$; $n_{solv,\lambda}$ = 1.3663 (e.g., λ = 273 nm; 25 °C). (B) Light waveguide flow cell of the same volume as part A. Equation 15 with NA = limited to, e.g., 0.22. (C) Typical light conductivity range of spectrophotometric HPLC detectors.

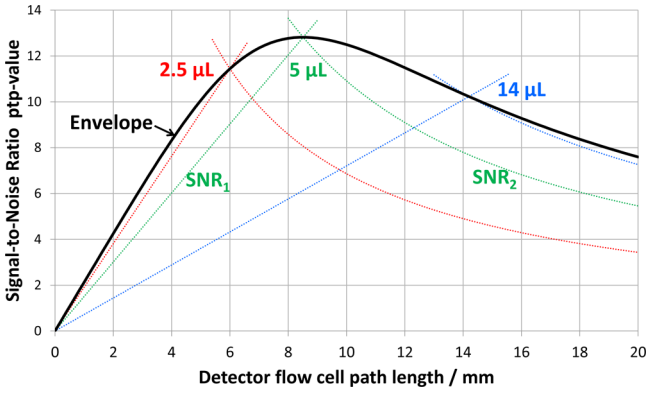

Figure 3. SNR_{ptp} of conventional cylindrical flow cell = f (L_{cell}): derivation/construction of SNR envelope curve for conventional detector flow cell; eqs 16 and 17. Parameters used to illustrate the approach: Optics: λ= 273 nm; $M_\lambda = 1 \times 10^{15}$ 1/(s sr cm^2 nm); $\Delta\lambda$ = 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 L/(mol cm). Chromatography: σ_{col}^2 = 5 μL^2 (e.g., d_{col} = 2.1 mm; L_{col} = 50 mm; d_p = 1.8 μm; N = 10 000; ε_{tot} = 0.52; k = 1.5). X = 5.

acceptable. The light pipe concept allows in particular the construction of low volume flow cells with a long path length at reasonable light throughput.

Optimal Flow Cell Path Length and Volume for Best Signal-to-Noise Ratio. Assuming extreme values for the optical path length and cell volume in eq 12, i.e., zero or infinite, will result in an SNR of zero. Hence there must be an optimal path length and optimal cell volume for the best SNR for every given chromatographic condition.

$$L_{cell} \to 0 \Rightarrow SNR \to 0 \quad \text{or} \quad L_{cell} \to \infty \Rightarrow SNR \to 0$$

$$V_{cell} \to 0 \Rightarrow SNR \to 0 \quad \text{or} \quad V_{cell} \to \infty \Rightarrow SNR \to 0$$

Baumann[19] emphasizes the importance of matching the light conductivity of the detector flow cell, as best as possible, to that of the optical system in order to reach an optimum for the minimum detectable optical density. Part of the following SNR optimization strategy for spectrophotometric HPLC detectors will also be based on this paradigm.

SNR Considerations for Conventional Detector Flow Cell. The starting point for the calculation of the flow cell path length and volume for best SNR is eq 12. For simplification reasons, all secondary parameters are pooled together to one factor f (see the List of Symbols). The following case differentiations are made.

Case 1: The detector optics is limiting light throughput. Then $G = G_{optics}$ and using eq 6 in 12 yields eq 16. SNR_1 is proportional to the flow cell path length L_{cell}.

$$SNR_1(L_{cell}) = f_1 \sqrt{\frac{G_{optics}}{\sigma_{\nu_i,col}^2 + \frac{V_{cell}^2}{X}}} L_{cell} \tag{16}$$

Case 2: The detector flow cell is limiting light throughput. Then $G = G_{conv\text{-}cell}$ and using eqs 6 and 14 in eq 12 yields eq 17: SNR_2 is inversely proportional to the flow cell path length L_{cell}.

$$SNR_2(L_{cell}) = f_2 \frac{V_{cell}}{\sqrt{\sigma_{\nu_i,col}^2 + \frac{V_{cell}^2}{X}}} \frac{1}{L_{cell}} \tag{17}$$

The SNR curves for different flow cell volumes are shown in Figure 3. The straight dotted lines represent case 1 where the light conductivity is limited by the detector optics. The curved dotted lines represent case 2 where the light conductivity is limited by the flow cell. Each corresponding intercept point represents a local optimum for a given flow cell volume and is the point where the light conductivity of the flow cell equals that of the optics. Connecting all intercept points yields the SNR envelope curve. The envelope curve has a clear optimum which delivers, to begin with, the path length for the best possible SNR for a given chromatographic condition.

Solving the parallel eqs 16 and 17 for the path length L_{cell} yields the path length at the intercept eq 18 which also follows from eq 14 alternatively:

$$L_{cell\text{-}at\text{-}intercept} = \sqrt[4]{\frac{f_{geo}\, n_{solv,\lambda}^2 V_{cell}^2}{G_{optics}} \Omega_0} \tag{18}$$

At the intercept, SNR_1 equals SNR_2, that is to say eq 16 equals eq 17. Using the path length at the intercept eq 18 in either eq 16 or 17 allows the description of the SNR envelope curve as a function of the flow cell volume eq 19. Secondary parameters are pooled together in factor f_3.

$$SNR_{conv\text{-}cell,ptp}(V_{cell}) = f_3 \sqrt{\frac{V_{cell}}{\sigma_{\nu_i,col}^2 + \frac{V_{cell}^2}{X}}} \tag{19}$$

The SNR has a clear optimum for the flow cell volume (Figure 4) which depends solely on chromatographic parameters and the dispersion behavior of the flow cell. The optimal flow cell volume for best SNR is given by

$$V_{cell\text{-}opt\text{-}SNR} = \sqrt{X}\,\sigma_{\nu_i,col} \tag{20}$$

It is advantageous to optimize the dispersion behavior of the detector flow cell. The less the flow cell contributes to zone dispersion, the larger its volume can be in the interest of improved light throughput. From eq 20 follows eq 21, the recommendation to match the variance of the detector flow cell to the column peak variance for the best SNR.

$$\sigma_{\nu,cell\text{-}opt\text{-}SNR}^2 = \sigma_{\nu_i,col}^2 \tag{21}$$

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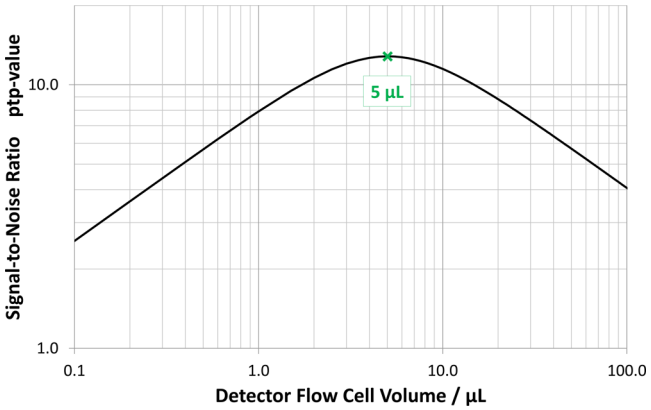

Figure 4. SNR of conventional detector flow cell = $f(V_{cell})$: eq 19. Optics: λ = 273 nm; $M_\lambda = 1 \times 10^{15}$ 1/(s sr cm^2 nm); $\Delta\lambda$ = 4 nm; G_{optics} = 0.03 mm^2 sr; T_{optics} = 0.14; η_λ = 0.25; τ = 1 s. Sample: caffeine in water; c_0 = 30 pg/μL; V_{inj} = 1.0 μL; Q_{inj} = 154.5 fmol; ε_λ = 9900 L/(mol cm). Chromatography: σ_{col}^2 = 5 μL^2 (e.g., d_{col} = 2.1 mm; L_{col} = 50 mm; d_p = 1.8 μm; N = 10 000; ε_{tot} = 0.52; k = 1.5). X = 5.

This is similar to and can be compared with the concept of impedance matching[25] in electronic design, in which the complex source impedance is matched to the load impedance for maximum power transfer with the difference that the contributions of the connecting lines in HPLC are not always as negligible as in electronics. The derivation of the conditions for best SNR in spectrophotometric HPLC detection is an important step toward a mathematical model for the relationship of chromatographic resolution and SNR. However, it should not be taken for granted that the conditions for best SNR are the best conditions for the chromatography as will be shown later. The SNR eq 19 can be normalized and expressed as a function of the ratio of the detector flow cell variance to the column peak variance of the chromatographic system eq 22. The "scaling" of the SNR curve in X and Y in eq 23 reduces the SNR characteristic to the ratio of two variances and will later allow to establish the relation between chromatographic peak resolution and SNR performance.

$$Z = \frac{\sigma_{\nu,cell}^2}{\sigma_{\nu_i,col}^2} = \frac{\sigma_{t,cell}^2}{\sigma_{t_i,col}^2} \quad (22)$$

$$Z \in \mathbb{R}_0^+$$

$$SNR_{rel} = \sqrt{\frac{2\sqrt{Z}}{1+Z}} \quad (23)$$

$$SNR_{rel} \in [0;\ 1]$$

SNR Considerations for Light Waveguide Detector Flow Cell. The same case differentiations as for conventional flow cells apply for light waveguide detector flow cells. Because the effect of solvent absorption on the overall transmission of the optics is not always negligible, in particular for longer light waveguide flow cells and in the low UV, an additional transmission factor $T_{solv,\lambda}$ described by eq 24 is introduced. α_λ is the solvent absorption coefficient at wavelength λ .

$$T_{solv,\lambda} = e^{-\alpha_\lambda L_{cell}} \quad (24)$$

Case 1: Detector optics is limiting light throughput and is described again by eq 16.

Case 2: The light waveguide detector flow cell is limiting light throughput. Then $G = G_{TIR\text{-}cell}$ and using eqs 6 and 15 and eq 24 in eq 12 yields eq 25. Secondary parameters are pooled together in factor f_4.

$$SNR_{TIR}(L_{cell}) = f_4 \sqrt{\frac{V_{cell}}{\sigma_{\nu_i,col}^2 + \frac{V_{cell}^2}{X}}} \sqrt{L_{cell}} \sqrt{e^{-\alpha_\lambda L_{cell}}} \quad (25)$$

SNR considerations for light waveguide flow cells then no longer result in a clear instruction for an optimal path length; see Figure 5. Because the signal is increasing faster than the noise, SNR continues to increase as the square root of the path length L_{cell} as long as a loss in transmission due to solvent absorption is negligible (i.e., α_λ near zero) (B). From this follows the design instruction to make light waveguide flow cells long rather than short for the best SNR. In cases of significant solvent absorption, e.g., when TFA is added to the solvents as a modifier, SNR starts to drop noticeably and the use of a shorter light waveguide flow cell will be the better choice (C). The SNR eq 25 for a light waveguide flow cell reveals also the concept of volume variance matching, as can be seen in the first of the square root expressions, but with a relatively free choice in path length.

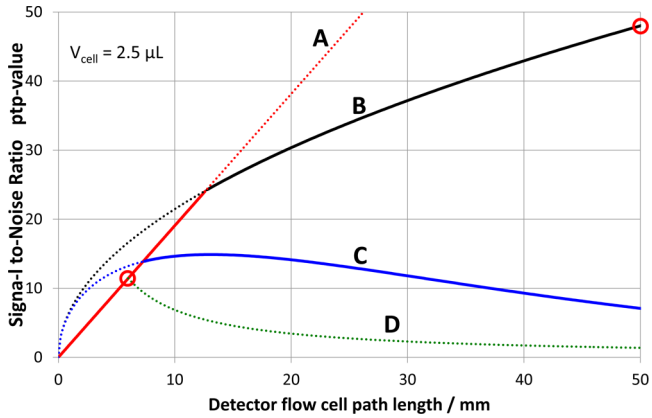

Figure 5. SNR of light waveguide flow cell = f (L_{cell}): (A) Light throughput limited by optics; eq 16. (B) Solvent absorption negligible, α_λ = 0 cm^{-1}; eq 25. (C) Solvent absorption not negligible, α_λ = 0.765 cm^{-1}; eq 25. (D) Conventional cylindrical flow cell of same volume; eq 17. Optics: λ = 273 nm; $M_\lambda = 1 \times 10^{15}$ 1/(s sr cm^2 nm); $\Delta\lambda$ = 4 nm; G_{optics} = 0.03 mm^2 sr; T_{optics} = 0.14; η_λ = 0.25; τ = 1 s; NA = limited to, e.g., 0.22. Sample: caffeine in water; c_0 = 30 pg/μL; V_{inj} = 1.0 μL; Q_{inj} = 154.5 fmol; ε_λ = 9900 L/(mol cm). Chromatography: σ_{col}^2 = 5 μL^2 (e.g., d_{col} = 2.1 mm; L_{col} = 50 mm; d_p = 1.8 μm; N = 10 000; ε_{tot} = 0.52; k = 1.5). X = 5.

At this point it makes sense to make a few important comments about consequences for the linear dynamic range[20] (LDR) of a spectrophotometric HPLC detector, which is the ratio of the highest detectable concentration c_{max} to the minimum detectable concentration c_{min} (limit of detection). For conventional flow cells, the optimization of SNR automatically leads to the best LDR. The concept of light waveguide flow cell allows for the construction of longer flow cells at the same volume for the same LDR compared to conventional flow cells. However, for a given flow cell volume, the design instructions for best SNR (long) and best LDR (short) are in direct opposition to each other. As SNR is improving as the square root of path length for light waveguide flow cells, LDR is decreasing to the same degree. The requirements for large LDR

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may be another reason, beside solvent absorption, to limit the path length of a light waveguide flow cell.

Theoretical Relation between Chromatographic Resolution and SNR. What is the loss in chromatographic resolution when optimizing the detector flow cell for the best SNR in spectrophotometric HPLC detection? What is the loss in SNR when setting a limit for the loss in chromatographic resolution?

Chromatographic resolution R_s is defined as the difference in retention time Δt_R of two adjacent peaks divided by the average peak width. The average peak width w_b is approximated by one of the two peak widths, which in turn is expressed by 4 times the standard deviation σ_t (here expressed in time units) for a peak of Gaussian shape.

$$R_s = \frac{t_{R_2} - t_{R_1}}{\left(\frac{w_{b_2} + w_{b_1}}{2}\right)} = \frac{\Delta t_R}{w_b} = \frac{\Delta t_R}{4\sigma_t} \tag{26}$$

The measured peak resolution $R_{s,tot}$ is always lower than the column peak resolution $R_{s,col}$ because every extra column volume leads to additional peak dispersion.

$$0 < R_{s,tot} < R_{s,col} \tag{27}$$

The relative "loss in resolution" r_{loss} is calculated using eq 28.

$$r_{loss} = \frac{R_{s,tot} - R_{s,col}}{R_{s,col}} \tag{28}$$

with

$$r_{loss} \in (-1; 0)$$

Under the assumption that the separation column and the detector flow cell are again the dominant sources of dispersion, which is always the desired case, the loss in resolution can also be expressed as a function of Z, the ratio of the detector flow cell variance to the column peak variance:

$$r_{loss} = \frac{1}{\sqrt{1 + Z}} - 1 \tag{29}$$

$$Z \in \mathbb{R}_0^+$$

Equations 23 and 29 allow the coupling of the SNR performance to the "loss in resolution".

$$\mathrm{SNR}_{rel} = \sqrt{2(1 + r_{loss})\sqrt{1 - (1 + r_{loss})^2}} \tag{30}$$

Figure 6 illustrates the relation between SNR and chromatographic resolution. The extreme values $r_{loss} = -1$ (no resolution at all) and $r_{loss} = 0$ (zero detector flow cell volume) result in an SNR of zero. Optimizing the SNR performance for any desired value of retention factor k (isocratic or gradient mode) by matching the variance of the detector flow cell to the column peak variance ($Z = 1$) results in a "Loss in Resolution" of −30% (see red arrows in Figure 6). In practice, of course, only the right-hand section of the SNR curve is of relevance. This is the "Arc of Compromises" to be made on which there is only a small window for good flow cell designs. It is typically better to accept a loss of −10% to −25% in SNR from the theoretical best value the optics (and the chromatography) can deliver while keeping the loss in chromatographic resolution within a reasonable limit of −10% to −5%. More strict limits for the loss in resolution involve drastic losses in SNR. The SNR that can be reached in practice depends on the molar extinction coefficient of the sample, the amount of sample injected, the optical output of the light source and on detector settings like spectral bandwidth and response time; refer to eq 12.

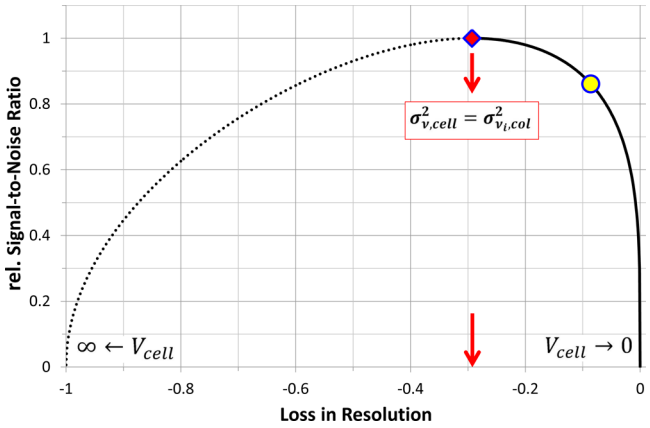

Figure 6. Relation between SNR and loss in resolution: eq 30. The circular marker represents an example data point for a 2.5 μL flow cell with a variance of 1.0 μL^2 that is working together with a 2.1 mm × 50 mm column; $\sigma_{col}^2 = 5\ \mu L^2$; (e.g., $d_p = 1.8\ \mu m$; $N = 10\,000$; $\varepsilon_{tot} = 0.52$; $k = 1.5$).

Because the resolution deteriorates relatively quickly with increasing flow cell volume and with small gains only in SNR, it is recommended to dimension the flow cell according to resolution requirements. A good starting point for the calculation of the flow cell volume is eq 31 that is derived from eq 29. For practical reasons (sample, solvent, and matrix and artifacts from the system), it is not useful to optimize the flow cell volume for separations below $k = 1$. A good and achievable value for the dispersion factor of the flow cell is, e.g., $X = 5$.

$$V_{cell} = \sigma_{v_i,col}\sqrt{X\left(\frac{1}{(1 + r_{loss})^2} - 1\right)} \tag{31}$$

CONCLUSIONS

A new quantitative model with the calculation of the trade-off made between chromatographic resolution and SNR in spectrophotometric HPLC detection is presented. The model recommends, and confirms the correctness of years of practical experience, that the detector flow cell should be preferably dimensioned according to resolution requirements rather than to best SNR. Ultimately a compromise needs to be made. The proposed model can help in finding the best compromise between resolution and SNR. It is advantageous to understand and improve the detector flow cells dispersion behavior because it allows increased flow cell volume and hence helps to improve the SNR performance. Light waveguide flow cells have, by principle, SNR and LDR performance advantages over conventional flow cells. Their unique properties make them particularly better flow cell candidates for low flow rate and low volume spectrophotometric HPLC detection.

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Notes

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■ LIST OF SYMBOLS

Roman Symbols

$A_{i,\lambda}$ [AU]	Absorbance measured at wavelength λ
c_i [mol L^{-1}]	Concentration of solute i
d_{col} [mm]	Internal diameter of separation column
d_p [μm]	Diameter particle stationary phase
f_{geo} [ ]	Geometrical form factor of conventional detector flow cell
G [mm^2 sr]	Light conductivity of optical system
k [ ]	Retention factor
L_{cell} [cm]	Detector flow cell path length = path length of absorption
L_{col} [mm]	Length of separation column
LDR_λ [ ]	Linear dynamic range of detector at wavelength λ
$m_{0,\lambda}$ [s^{-1}]	Photon flux at balance; t = 0
m_λ [s^{-1}]	Reduced photon flux caused by sample absorption
M_λ [1/(s sr mm^2 nm)]	Spectral photon flux density of detector light source
N [ ]	Column plate number
NA [ ]	Numerical aperture of TIR-FC NA = $(n_{solv}^2 - n_{clad}^2)^{1/2}$
N_{ptp} [AU]	Peak-to-peak value of noise
N_{rms} [AU]	Root-mean-square value of noise
$n_{solv,\lambda}$ [ ]	Refractive index of solvent at wavelength λ
$n_{clad,\lambda}$ [ ]	Refractive index of the light waveguide clad at λ
$Q_{inj,i}$ [mol]	Sample amount injected
r_{loss} [ ]	Loss in resolution $\in$ (−1,0)
R_s [ ]	Resolution
SNR_{ptp} [ ]	Peak-to-peak value of signal-to-noise ratio
$T_{optics,\lambda}$ [ ]	Overall transmittance of detector optics
t_R [min]	Retention time
$T_{solv,\lambda}$ [ ]	vTransmission of solvent at wavelength λ
V_{cell} [μL]	Volume of detector flow cell
$V_{R,i}$ [μL]	Retention volume $V_{R,i} = (\pi/4)\, d_{col}^2 L_{col} \varepsilon_{tot}(1 + k)$
V_{inj} [μL]	Injection volume
w_b [min]	Peak width (measured at base)
X [ ]	Dispersion factor of detector flow cell $\in$ [1;12]
Z [ ]	Variance ratio

Greek Symbols

α_λ [cm^{-1}]	Absorption coefficient of solvent at wavelength λ
$\varepsilon_{i,\lambda}$ [L mol^{-1} cm^{-1}]	Molar absorptivity of solute i
ε_{tot} [ ]	Columns total porosity
η_λ [ ]	Quantum efficiency of photo detector
$\Delta\lambda$ [nm]	Spectral bandwidth of detector
τ [s]	Response time of detector
$\sigma_{v,tot}^2$ [μL^2]	Total variance of measured peak
$\sigma_{v,inj}^2$ [μL^2]	Variance of sample injector
$\sigma_{v,col}^2$ [μL^2]	Variance of separation column
$\sigma_{v,cap}^2$ [μL^2]	Variance of connection tubing
$\sigma_{v,cell}^2$ [μL^2]	Variance of detector flow cell
$\sigma_{t,col}^2$ [s^2]	Variance of separation column expressed in time units
$\sigma_{t,cell}^2$ [s^2]	Variance of detector flow cell expressed in time units
τ [s]	Response time of detector
Ω_0 [sr]	Standard unit of solid angle; Steradian

Proportional Factors

$$f_1 = \frac{2.3}{6\sqrt{2\pi}} \varepsilon_{i,\lambda} Q_{inj,i} \sqrt{M_\lambda \Delta\lambda T_{optics,\lambda} \eta_\lambda \tau}$$

$$f_2 = f_1 \sqrt{f_{geo}\, n_{solv,\lambda}^2 \Omega_0} \quad \text{e.g.,} f_{geo} = f_{geo,cyl} = \frac{10}{3}$$

$$f_3 = f_1 \sqrt[4]{f_{geo}\, n_{solv,\lambda}^2 \Omega_0 G_{optics}}$$

$$f_4 = \frac{2.3}{6\sqrt{2}} \varepsilon_{i,\lambda} Q_{inj,i} \sqrt{M_\lambda \Delta\lambda NA_{TIR\text{-}cell}^2 \Omega_0 T_{optics,\lambda} \eta_\lambda \tau}$$

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