

Where Has My Efficiency Gone? Impacts of Extracolumn Peak Broadening on Performance, Part 1: Basic Concepts

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1 Where has my efficiency gone? Impacts of extra-column peak broadening
2 on performance, Part I – Basic concepts

3 Dwight R. Stoll and Ken Broeckhoven

4

5 [keywords]

6 Extra-column band broadening, dispersion, peak broadening, resolution, tubing, injection,
7 detection

8

9 [teaser]

10 Dispersion (broadening, or spreading) of analyte zones (peaks) outside of chromatography
11 columns can seriously erode the resolution provided by good columns. Understanding the
12 relationship between the level of dispersion associated with a given instrument relative to the
13 intrinsic efficiency of a given column can help avoid disappointing results, especially when
14 switching to new column technologies. In this installment we focus on basic concepts in extra-
15 column dispersion as a prelude to future installments that will focus on dispersion in specific
16 elements of HPLC systems.

17

18 [main text]

19 Even from the very early days of the development of chromatography ‘systems’ that provided for
20 mechanized introduction of samples into the mobile phase flow path it was recognized that
21 dispersion of analyte zones outside of the separation column could compromise the quality of the
22 separation as measured by resolution [1]. During the 400-bar era of liquid chromatography that
23 lasted through the mid-2000s, instrument manufacturers worked to adjust the dimensions of
24 component parts including connecting tubing and detector elements so that the impact of
25 dispersion of the analyte zone outside of the column was manageable and did not seriously affect
26 separation performance in most cases. However, the introduction of instruments capable of
27 separating at much higher pressures (for example, 1000 bar and beyond) starting in the mid-
28 2000s has precipitated a dramatic growth in the use of narrower columns (mostly 2.1 mm i.d.)
29 prepared with smaller particles (that is, moving below three micron). When used under these
30 conditions, these columns have the potential to produce peaks with much lower volumes (on the

31 order of 10 times or more) compared to those that were typical in the 400-bar era. These lower
32 volume peaks are, in turn, more susceptible to dispersion outside of the column to a degree that
33 can seriously compromise the quality of the separation. As a result, there has been renewed
34 interest in the topic of extra-column dispersion (ECD) in the last 15 years, both from instrument
35 manufacturers looking to improve the performance of their instruments along these lines, and
36 from researchers looking to further our understanding of these issues so that we can leverage
37 this knowledge in the development of better methods [2]. So, in spite of the fact that ECD has
38 been studied intensively since the very early days of HPLC, a lot has changed recently. For this
39 month's installment of LC Troubleshooting I have asked Dr. Ken Broeckhoven – fellow LCGC
40 Emerging Leader, and one of the world's experts on ECD - to join me in writing a multi-part series
41 on this topic where we aim to translate new findings described in the chromatographic literature
42 into terms that can be applied in development and troubleshooting of modern HPLC methods. In
43 this first installment we begin by reviewing terms and concepts that are central to the discussion
44 and quantify the effects of ECD on the performance of separations executed using columns of
45 different dimensions and prepared with particles of different sizes. In subsequent parts of this
46 series we will go into more detail discussing how different parts of HPLC systems (for example,
47 injectors, detectors, and connecting tubing) contribute to the overall dispersion that ultimately
48 impacts the quality of a separation. We are hopeful that an improved understanding of these
49 factors will help users identify situations where ECD may be unnecessarily compromising the
50 quality of their separations, and identify a viable path to remedy this type of situation.

51 ~ Dwight Stoll

52

53 **Getting acquainted with the problem**

54 Given that the pace of change in HPLC column market has been rapid recently, and that the
55 turnover of HPLC instruments in laboratories is relatively slow, it is commonplace for users to try
56 out a new column technology using a relatively old instrument. Sometimes this works just fine,
57 and sometimes it does not. We'd really like to avoid situations where the apparent column
58 performance does not measure up to expectations, not because the columns doesn't work
59 properly, but because the influence of the instrument on the outcome obscures the actual column
60 performance. Many column manufacturers have recognized the seriousness of this problem, and
61 invested substantially in the development of educational material, tutorials and trainings, and

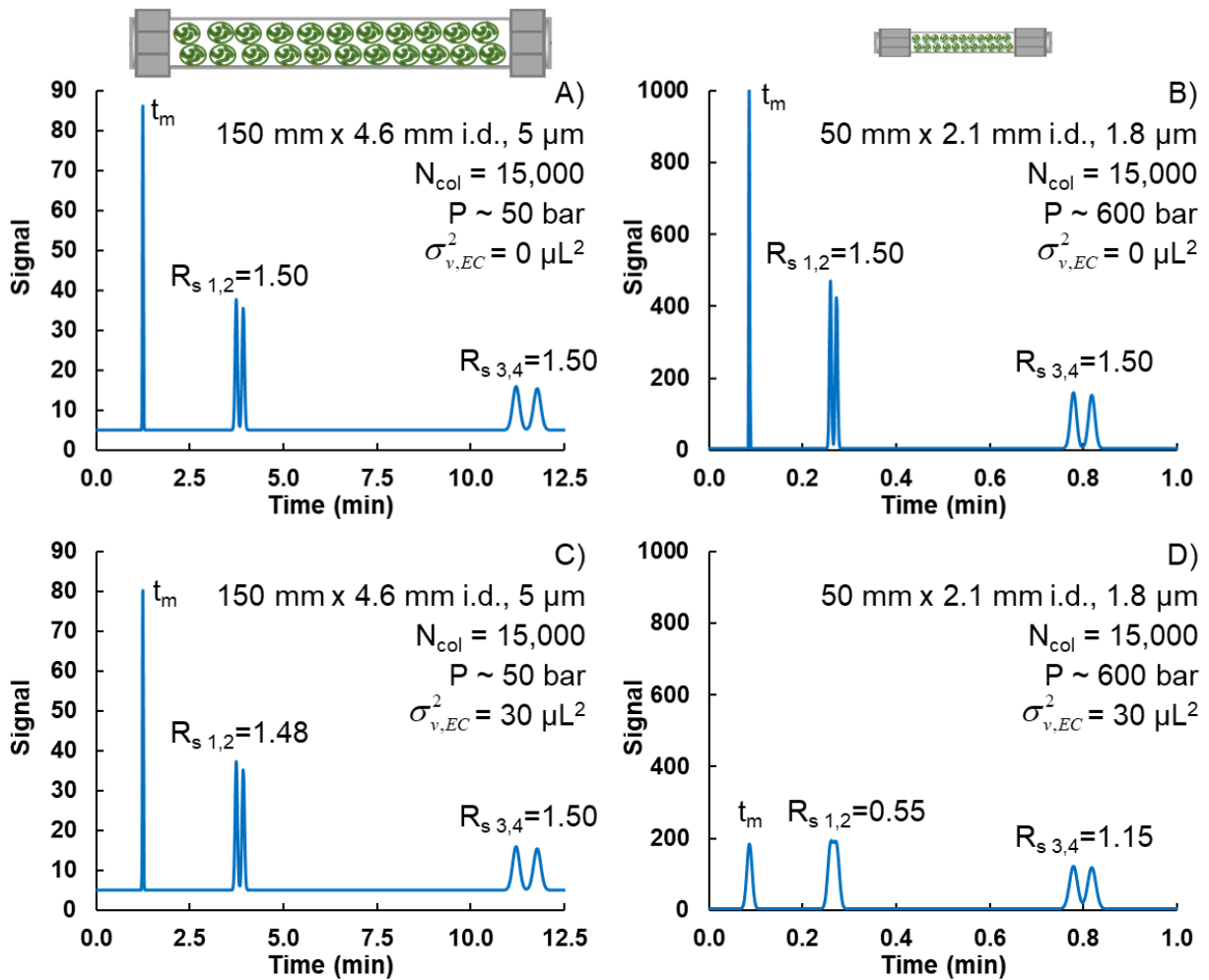
62 software (for example, method transfer calculators) to help users avoid bad experiences with new
63 column technologies.

64 We recognize that many practicing chromatographers think in terms of chromatograms. To get
65 acquainted with the problem we're discussing here in a quantitative way, we've prepared the
66 simulated chromatograms shown in Figure 1, which illustrates the impact of ECD typical of an
67 older HPLC instrument on the quality of separations obtained with relatively older or newer column
68 technologies. Panels A and B show simulated chromatograms for isocratic separations of a
69 hypothetical five-component mixture of compounds with retention factors ranging from 0 to 8.5.
70 Panel A shows the separation of the mixture using a 150 mm x 4.6 mm i.d. column packed with
71 5 μm particles, operated at a mobile phase flow rate of 1 mL/min, where we assume that there is
72 absolutely no influence of dispersion outside of the column on the observed chromatogram (that
73 is, $\sigma_{v,EC}^2 = 0$; this is completely hypothetical, but useful to support discussion). The separation is
74 good with a minimum resolution of 1.5 for both compound pairs 1/2 and 3/4, and the analysis is
75 complete in about 12 minutes. Now, for a separation on this timescale, one of the potential
76 advantages of moving to a column with a smaller particle size is that the same resolution achieved
77 with larger particles (Panel A) can be achieved in a shorter time, provided that the pressure
78 required to drive the separation using smaller particles can be generated by the pump. Panel B
79 shows what such an improvement could look like. We see that moving to a shorter column packed
80 with smaller particles yields the same resolution in a much shorter time provided that the flow rate
81 is similar to what was used for the column with larger particles. Again, this assumes there is no
82 impact of the dispersion outside of the column on resolution, and it is critically important to
83 recognize here that this degree of improvement can only be realized if a much higher pressure is
84 available.

85 Things become more interesting when we consider cases where dispersion outside of the column
86 *does* impact resolution. By comparing Panels A and C, and B and D, we can see that the
87 magnitude of the impact of ECD is strongly influenced by the column in use. In the case of the
88 long column with large particles, the level of ECD chosen here (which reflects the characteristics
89 of a 'good' system from the end of the 400-bar era; see more discussion below) *does not affect*
90 *the resolution of the 3/4 peak pair at all*. There is a very small affect on the resolution of the 1/2
91 peak pair, but this level of change certainly would not be detectable by eye. In other words,
92 separations involving long columns and large particles tend to be relatively unaffected by
93 dispersion outside of the column. In Panel D, however, we see a completely different outcome. If
94 we take the shorter, narrower column packed with smaller particles and carry out the separation

95 using the same instrument characterized by an extra-column variance of $30 \mu\text{L}^2$ as in Panel C,
 96 the system has a devastating negative impact on the quality of the separation. The resolution of
 97 the 3/4 peak pair decreases by 23%; the *loss of resolution for the 1/2 peak pair is so bad that it is*
 98 *no longer even clear if there are actually two peaks present* at that point in the chromatogram.

99 So – coming back to the title of this installment – a perfectly good column can be robbed of its
 100 performance potential as a result of using it in an instrument that is not well suited to modern
 101 column technologies. In the remainder of this article, and in the other parts of this series, our aim
 102 is to provide an update on recent research results that inform our understanding of how different
 103 instrument parameters contribute to the level of ECD exhibited by different systems. We can then
 104 use this knowledge to troubleshoot problems originating from these effects, and build better
 105 methods from the start. Readers interested in learning much more about details related to this
 106 topic are referred to the recent review article by one of us [2], as well as recent contributions on
 107 the topic in LCGC Magazine [3–9].



109 **Figure 1.** Comparison of the effect of extra-column dispersion on resolution for separations using “large”
110 or “small” columns. Assumptions: Total column porosity, 0.5; Flow rate, 1.0 mL/min.; Plate heights, 10 μm
111 (150 mm column) and 3.3 μm (50 mm column); k values, 1) 2.00, 2) 2.15, 3) 8.00, 4) 8.45. The elution time
112 of an unretained compound is indicate by t_m .

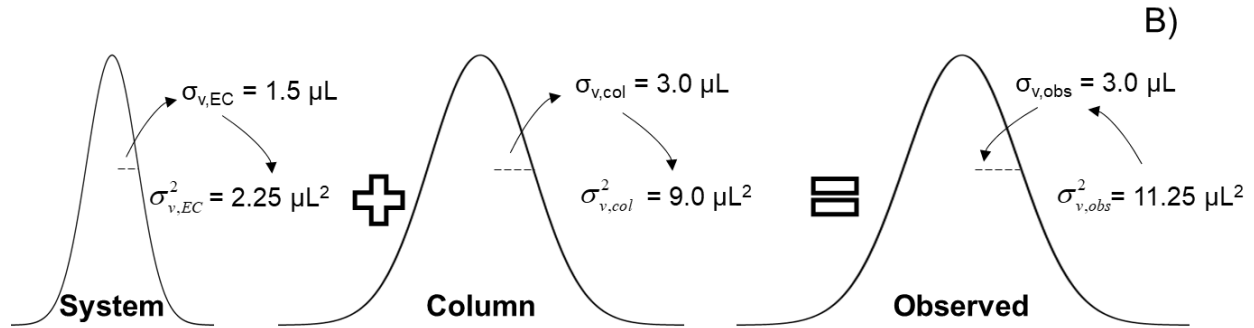
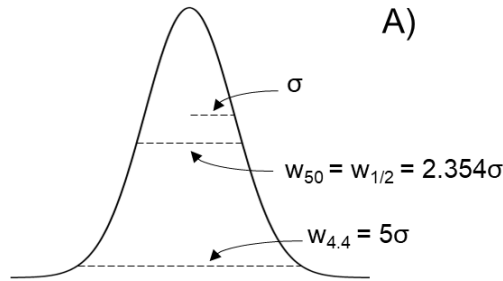
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114 **Extra-column dispersion – Measuring it, and adding it**

115 One of the simplest definitions of column efficiency is that it is calculated as the square of the ratio
116 of the the retention time (or volume) to the peak width (in time or volume units to match the
117 retention measurement). If the peak is symmetrical and has a Gaussian shape, then the simplest
118 calculation of the efficiency relies on the standard deviation (σ) of the Gaussian, shown in Figure
119 2A. It is common to measure the peak width at half of the peak height ($w_{1/2}$). In the case of a
120 Gaussian peak the relationship between $w_{1/2}$ and σ is straightforward and thus σ can be calculated
121 from $w_{1/2}$. When peaks are perfectly symmetrical, determining σ by measuring the peak width at
122 50% of the height (w_{50}) or 4.4% of the height ($w_{4.4}$) will yield exactly the same σ number.
123 However, as the peak becomes more asymmetric, the w_{50} value will underestimate the true peak
124 variance, and thus using $w_{4.4}$ provides a more conservative measure of the peak variance. More
125 rigorous determination of the peak variance of asymmetric peaks requires the use of statistical
126 moments, which comes with its own challenges. Readers interested in learning more about these
127 details are referred to the recent review of many issues related to ECD [2].

128 One of the simplest approaches to determining the level of total ECD involves replacing the
129 column with a zero-dead volume union and injecting a sample. The width of this “system peak”
130 can then be determined using one of the methods discussed above. The most critical step in
131 assessing the impact of the ECD on the observed column efficiency is recognizing that it is the
132 variances (σ^2) of system and column peaks that are additive, not the widths (that is σ , determined
133 by any of the methods above). The process of estimating the impact of the extra-column variance
134 on the observed column efficiency is shown graphically in Figure 2. The standard deviations of
135 the system and column peaks are first measured or calculated, the variances for each component
136 are calculated, and then added together. The resulting variance expected for the observed peak
137 can then either be used as is, or converted back to a peak width.

138



139

140 **Figure 2.** Illustration of different ways of determining the peak standard deviation (σ) (A), and the concept
 141 of addition of peak variances to assess the impact of extra-column dispersion on observed column
 142 efficiency (B).

143

144 **Impact of extra-column dispersion on observed column efficiency over a range of**
 145 **conditions**

146 In Figure 1 we illustrated the effect of one level of extra-column variance on the resolution
 147 provided by two columns of very different dimensions. It is also instructive to examine the
 148 magnitude of this effect over ranges of retention factors, column dimensions and particle sizes,
 149 and extra-column variance levels. The results of this kind of calculation provide a kind of map that
 150 one can use to estimate the seriousness of the effect of the extra-column effect when approximate
 151 values of retention factor, extra-column variance, and column dimensions are known. Whereas in
 152 Figure 1 we used the resolutions of specific peak pairs to quantify the loss of performance, in the
 153 following discussion we will use the loss of column efficiency as a metric, because provides a more
 154 generalizable view of the problem.

155 There are a number of ways one could approach these calculations that vary in the level of rigor
 156 and accuracy required, but we've taken the following approach to arrive at the numbers shown in
 157 Figure 3. We start by estimating the inherent efficiency of the column (N_{col}) (that is, without extra-

158 column effects) from the column length (L), particle size (d_p), and the reduced plate height (h). In
 159 our case here we've assumed that the reduced plate heights for fully porous particles (Figures 1A
 160 and 1C) are 2.0, and 1.5 for superficially porous particles (Figure 1B). A more rigorous approach
 161 would require an estimate of the flow rate (F), and the dependence of h on the F through
 162 something like a van Deemter curve. We also note here that the following calculations and
 163 discussion are only relevant to isocratic separations. In future installments we will discuss how
 164 things change in the case of separations that use gradient elution.

$$165 \quad N_{col} = \frac{L}{H} = \frac{L}{h * d_p} \quad (1)$$

166 Once we have the column efficiency, we can calculate the standard deviation of a peak at any
 167 retention factor as long as we know the column dimensions and have an estimate of the total
 168 porosity of the column. A reasonable estimate for the total porosities (ϵ_T) of columns packed with
 169 fully and superficially porous particles is 0.5 [10]. First, the column dead volume (V_m) is calculated
 170 from the physical dimensions of the cylinder and the porosity.

$$171 \quad V_m = \pi * r^2 * L * \epsilon_T \quad (2)$$

172 Then, the retention volume (V_R) can be calculated for a compound with retention factor k , and
 173 finally the peak standard deviation from the retention volume and the column efficiency.

$$174 \quad V_R = V_m (1 + k) \quad (3)$$

$$175 \quad \sigma_{v,col} = \frac{V_R}{\sqrt{N_{col}}} \quad (4)$$

176 Now, with the expected peak standard deviation in hand, and an estimate of the extra-column
 177 variance ($\sigma_{v,EC}^2$), we can calculate the standard deviation that will be observed at the detector
 178 ($\sigma_{v,obs}$), where this will be larger than $\sigma_{v,col}$ due to additional dispersion outside of the column.

$$179 \quad \sigma_{v,obs} = \sqrt{\sigma_{v,col}^2 + \sigma_{v,EC}^2} \quad (5)$$

180 The apparent column efficiency (N_{obs}) is then calculated by rearrangement of equation 4. Note
 181 that here we assume that V_R is unaffected by the system volume outside of the column. This most
 182 definitely is an approximation and a more rigorous approach would not rely on this assumption,

183 but this is a minor issue in the context of the parameters chosen here and the focus of this
184 discussion.

$$185 \quad N_{obs} = \left(\frac{V_R}{\sigma_{v,obs}} \right)^2 \quad (6)$$

186 Finally, the percent loss of column efficiency is calculated as:

$$187 \quad \%Loss = 100\% * \left(\frac{N_{obs} - N_{col}}{N_{col}} \right) \quad (7)$$

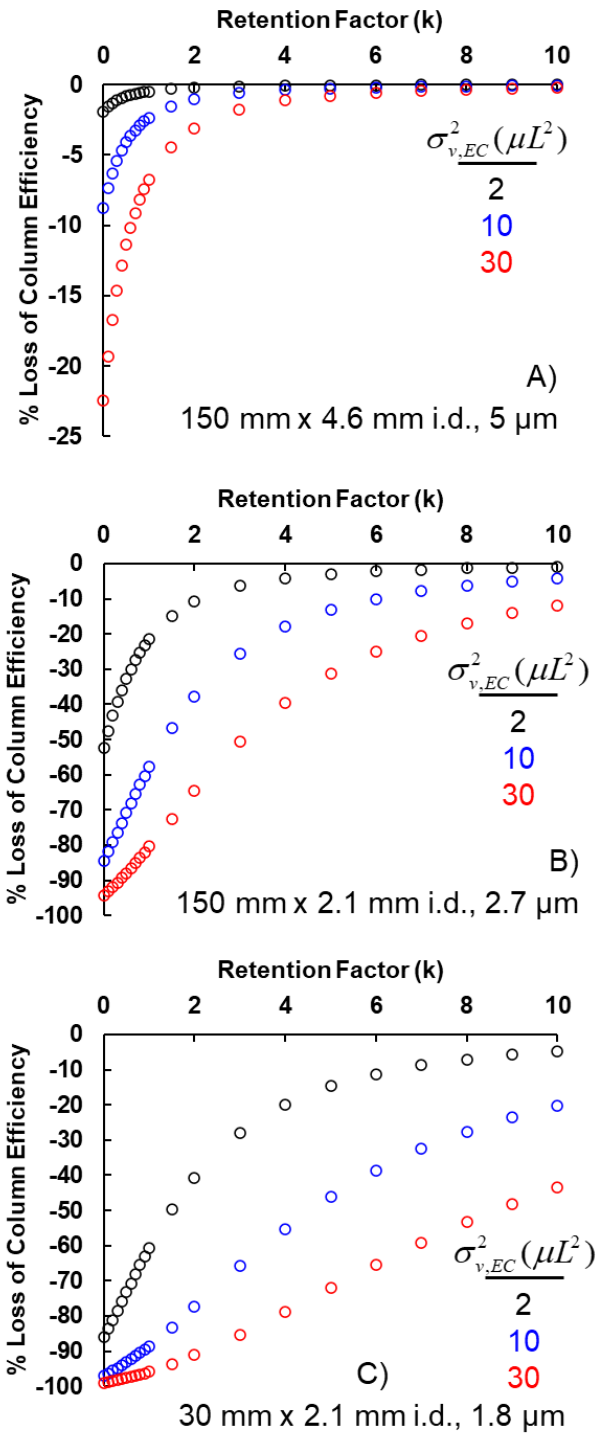
188 A series of results from this type of calculation are shown in Figure 3 for three different columns
189 and three levels of extra-column variance, over a range of retention factors from 0 to 10. The
190 three columns selected are very different, and represent: A) large columns (150 mm x 4.6 mm
191 i.d.) packed with large particles (5 μm) that are still often used in legacy methods that were
192 developed in the 400-bar era; B) long (150 mm), narrow (2.1 mm i.d.) columns packed with
193 superficially porous particles of intermediate size (2.7 μm) used for modern, high efficiency
194 separation; and C) short (30 mm), narrow (2.1 mm i.d.) columns packed with sub-2 μm particles
195 used for very fast 1D separations, or as the second dimension column in 2D-LC separations. The
196 three levels of extra-column variance selected represent the level of dispersion found in good
197 instruments from the end of the 400-bar era (30 μL^2), "off-the-shelf" configurations of modern
198 UHPLC instruments (10 μL^2), and highly optimized configurations of UHPLC instruments aimed
199 at minimizing extra-column dispersion (2 μL^2) [2].

200 There are many useful observations we can make in reviewing these results. First, as discussed
201 above, the performance of large columns like that shown in Panel A is relatively insensitive to
202 reasonable levels of extra-column variance. Even with the largest variance of $\sigma_{v,EC}^2 = 30\mu\text{L}^2$ the
203 maximum efficiency loss for any compound with $k > 1$ is only about 5%. Using this type of column
204 in an instrument with lower extra-column dispersion is not very helpful from the point of view of
205 the observed efficiency (N_{obs}), unless one is dealing with very low retention compounds with $k <$
206 1 (which is important in size exclusion chromatography [SEC], for example).

207 Moving to the results shown in Panel B for the 150 mm x 2.1 mm i.d. columns packed with 2.7
208 μm superficially porous particles, we see that using this type of column in an instrument from the
209 400-bar era will result in efficiency losses of more than 30% for compounds with $k < 5$. This
210 obviously would be a serious disappointment for someone trying a column like this for the first

211 time. Moving to an off-the-shelf UHPLC instrument helps quite a bit, but even in this case the
212 efficiency loss for compounds with $k < 1$ is more than 50%. Realizing the full potential of this
213 column requires some optimization of the UHPLC instrument. Reducing the extra-column
214 dispersion to $2 \mu\text{L}^2$ enables realization of more than 90% of the actual column efficiency for
215 compounds with $k > 2$.

216 Out of the scenarios considered here, the results in Panel C show that using short, narrow
217 columns packed with sub-2 μm particles in instruments without careful attention to the level of
218 extra-column dispersion will lead to disastrous results. Even with a highly optimized UHPLC
219 system, 80% of the actual column efficiency is only reached for compounds with $k > 4$. With
220 columns of these dimensions we must be very careful to manage our expectations for
221 performance by taking into account the characteristics of the instrument surrounding the column.



222

223 **Figure 3.** Calculated loss of column efficiency due to extra-column dispersion for different combinations of
 224 column dimensions, particle size, and level of extra-column variance, as a function of retention factor.
 225 Assumptions: $\epsilon_T = 0.5$; extra-column volume does not add significantly to the apparent retention volume.
 226 Columns: A) 150 mm x 4.6 mm i.d., 5 μm ($h = 2$); B) 150 mm x 2.1 mm i.d., 2.7 μm ($h = 1.5$); C) 30 mm x
 227 2.1 mm i.d., 1.8 μm ($h = 2$).

228

229

230 **Summary**

231 In this installment of LC Troubleshooting we have reviewed some of the basic concepts relevant
232 to the consideration of extra-column dispersion (ECD) that occurs in (U)HPLC systems, and the
233 impact of this dispersion on the apparent performance of columns of different dimensions and
234 efficiencies. As columns become smaller, and as they are packed with smaller particles, attention
235 to the minimization of ECD associated with the instrument becomes more important. Not
236 considering this can result in very poor apparent column performance that has nothing to do with
237 the actual column performance, and everything to do with robbing the column of its inherent
238 efficiency by dispersion of the analyte zone that occurs outside of the column.

239 Here we have talked about ECD from the point of view of an entire instrument. In upcoming
240 installments we will dig into the details of dispersion that occurs in different elements of these
241 systems, including injectors, detectors, connecting tubing, and when post-column flow splitting is
242 used, as well as differences in the treatment in of ECD with gradient elution is used.

243

244

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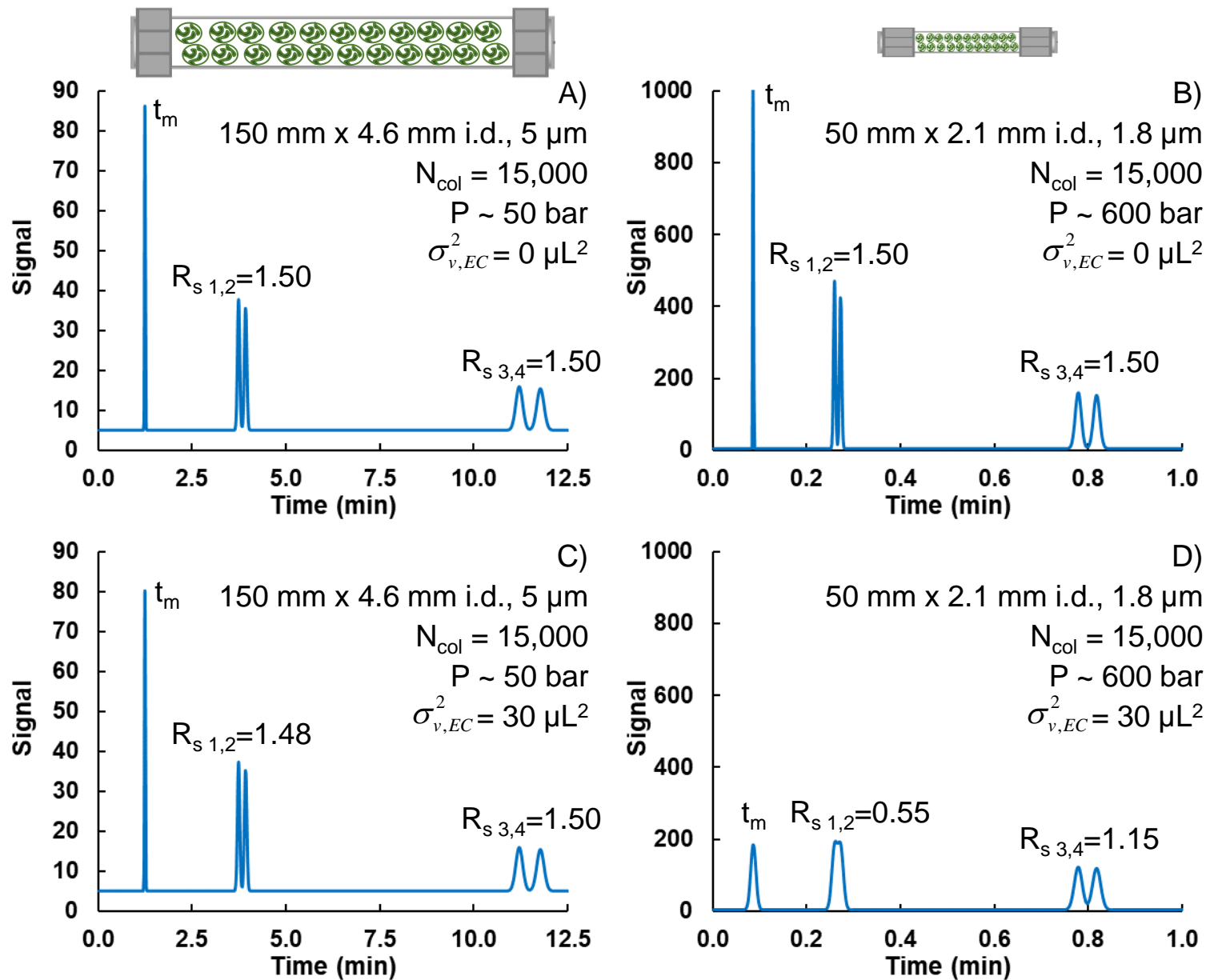


Figure 1. Comparison of the effect of extra-column broadening on resolution for separations using “large” or “small” columns. Assumptions: Total column porosity, 0.5; Flow rate, 1.0 mL/min.; Plate heights, 10 μm (150 mm column) and 3.3 μm (50 mm column); k values, 1) 2.00, 2) 2.15, 3) 8.00, 4) 8.45.

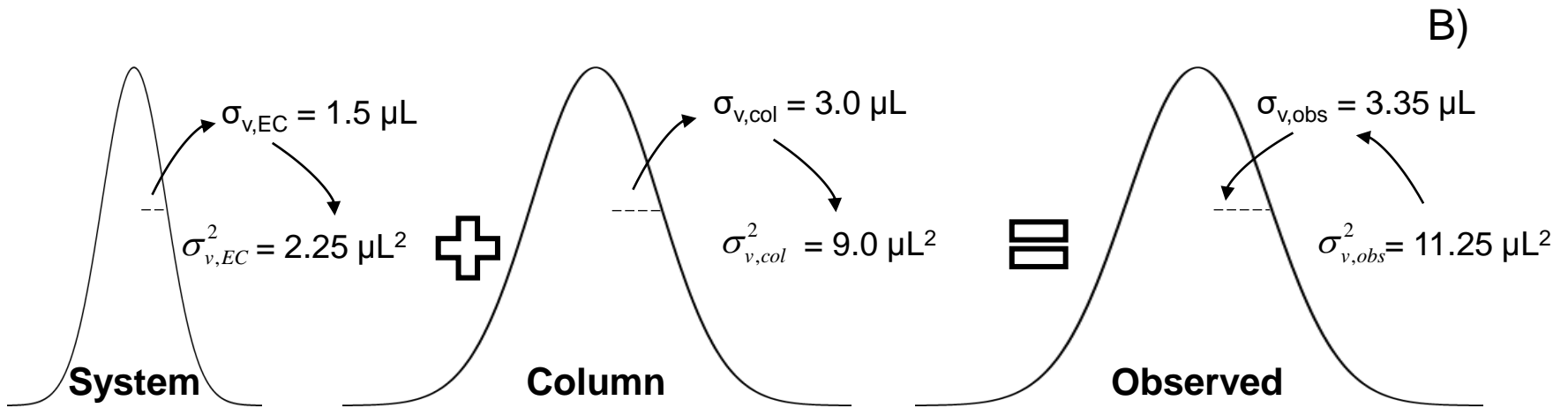
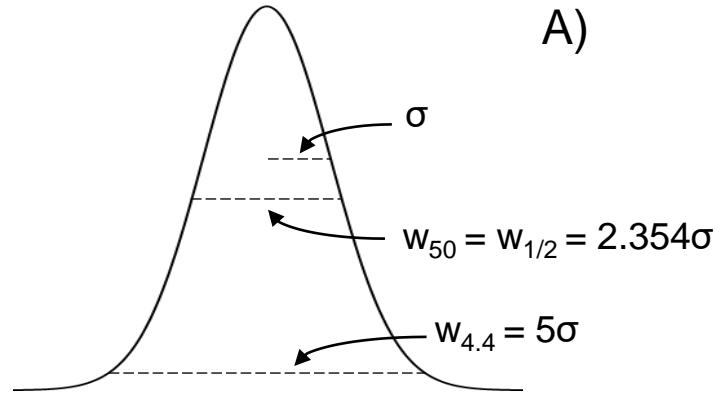


Figure 3. Calculated loss of column efficiency due to extra-column dispersion for different combinations of column dimensions, particle size, and level of extra-column variance, as a function of retention factor.

Assumptions: $\epsilon_T = 0.5$; extra-column volume does not add significantly to the apparent retention volume.

Columns: A) 150 mm x 4.6 mm i.d., 5 μm ($h = 2$); B) 150 mm x 2.1 mm i.d., 2.7 μm ($h = 1.5$); C) 30 mm x 2.1 mm i.d., 1.8 μm ($h = 2$).

