Implications of dispersion in connecting capillaries for separation systems involving post-column flow splitting

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Abstract

It is common practice in liquid chromatography to split the flow of the effluent exiting the analytical column into two or more parts, either to enable parallel detection (e.g., coupling the separation to two destructive detectors such as light scattering and mass spectrometry (MS)), or to accommodate flow rate limitations of a detector (e.g., electrospray ionization mass spectrometry). In these instances the user must make choices about split ratio and dimensions of connecting tubing that is used between the split point and the detector, however these details are frequently not mentioned in the literature, and rarely justified. In our own work we often split the effluent following the second dimension (^{2}D) column in two-dimensional liquid chromatography systems coupled to MS detection, and we have frequently observed post ^{2}D column peak broadening that is larger than we would expect to result from dispersion in the MS ionization source itself. For the present paper we describe a series of experiments aimed at understanding the impact of the split ratio and post-split connecting tubing dimensions on dispersion of peaks exiting an analytical column. We start with the simple idea – based on the principle of conservation of mass – that analyte peaks entering the split point are split into two parts such that the analyte mass (and thus peak volume) entering and exiting the split point is conserved, and directly related to the ratio of flow rates entering and exiting the split point. Measurements of peak width and variance after the split point show that this simple view of the splitting process – along with estimates of additional dispersion in the post-split tubing - is sufficient to predict peak variances at the detector with accuracy that is sufficient to guide experimental work (median error of about 10% over a wide range of conditions). We feel it is most impactful to recognize that flow splitting impacts apparent post-column dispersion not because anything unexpected happens in the splitting process, but because the split dramatically reduces the volume of the analyte peak, which then is more susceptible to dispersion in connecting tubing that would not cause significant dispersion under conditions where splitting is not implemented. These results will provide practitioners with a solid basis on which rational decisions about split ratios and dimensions of post-split tubing can be made.

1. Introduction

Dispersion of analyte peaks outside a chromatography column (i.e., extra-column dispersion) can seriously reduce the resolving power of a separation that would otherwise be excellent in the absence of such dispersion. These effects have been known for decades [1,2], but continue to be discussed in more and more depth as the inherent performance of column technologies continually improves [3–7]. With the move to sub-three micron particles we are observing a gradual shift toward widespread use of 2.1 mm i.d. columns. These narrow columns, when packed with small particles, can produce peak volumes on the order of a few microliters or less; under these conditions even the best high performing commercially available LC systems today can contribute substantially to the overall peak variance, and great care must be taken to optimize the performance of these systems [3–7].

In some applications there can be a mismatch between the flow rate that is optimal for the pre-detection components of the system (e.g., pump and column), and the flow rate that is optimal for the detector. An example of this is the coupling of two-dimensional liquid chromatography (2D-LC) separations to detection using mass spectrometry (MS) [8–10]. Whereas the flow rates in the second dimension (^{2}D) separations of comprehensive 2D-LC separations that are optimal for maximizing peak capacity tend to be in the 1-3 mL/min. range, optimal flow rates for most MS instruments for analytical scale LC (nano-flow LC uses much lower flow rates) tend to be in the range of 100-500 μL/min. [11,12]. It is common practice to split a portion of the column effluent and divert it to waste (or a different detector) while the remainder is directed to the MS detector [10,13–15]. This strategy allows the two components (the 2D-LC and the MS) of the system to be operated under optimal conditions simultaneously. Although sophisticated ‘active splitter’ devices are commercially available that allow users to continuously vary the post-column split ratio, in the literature the most commonly reported device used for such splitting is a simple tee-piece fitted with two restriction capillaries whose lengths are adjusted to give the desired split ratio.

In our own work where we frequently use UV absorbance spectroscopic and MS detection following the ^{2}D separation in 2D-LC systems we have consistently observed that peaks recorded at the MS detector are significantly wider than they are in the UV detector. In the process of trying to understand these observed differences in peak width, we have found that the dimensions of the capillaries connecting the tee-piece to the detector have a dramatic influence on the extent of peak dispersion observed under these conditions, in agreement with a report by Spaggiari et al. [16], and discussion by Hetzel et al. [17]. While previous studies have reported dispersion data for a variety of connecting capillaries for LC [3,16,18], we are not aware of a dataset that focuses on the particular capillary dimensions and mobile phase flow rates needed to optimize the flow splitting conditions of interest here. Therefore, this paper aims to quantify the dispersion that happens in the particular capillaries that could be used in such systems under flow splitting conditions, so that we can have a rational basis for choosing capillaries for this purpose. There is an extensive body of literature focused on both theoretical and experimental aspects of dispersion in connecting capillaries for chromatography [3,6,18–23]. Our purpose here is not to add to the theoretical understanding of dispersion of these capillaries *per se*. Rather, our primary aim here is to highlight the special circumstances (i.e., very small volumetric peak variances) that flow splitting gives rise to, present experimental dispersion data relevant to flow splitting conditions, and provide a framework that will enable users to choose optimal capillaries for their own systems without having to make their own dispersion measurements.

Our interpretation of our experimental results discussed below is based on the central assertion that an analyte peak entering one branch of a simple tee-piece is split into two parts exiting the other two branches of the tee-piece, and that – as required by the conservation of mass – the volume fraction of the peak exiting each branch is directly related to the split ratio. Assuming the tee-piece itself does not contribute to band broadening, the same can be said of the peaks' standard deviations (σ_v). This is illustrated explicitly in Fig. 1 for the case of a 1:4 split ratio; one-fifth of the peak volume entering the tee-piece from the analytical column ($\sigma_{v,col}$) proceeds toward the detector, and four-fifths of the peak volume proceed toward waste (or a different detector in some applications).

2. Experimental

2.1 Chemicals and reagents

Fluorescein sodium salt, acetophenone, ammonium acetate, and formic acid were obtained from Sigma-Aldrich (St. Louis, MO). Fluorescein was dissolved in HPLC grade water to prepare a stock solution at 10 mg/mL, and acetophenone was dissolved in acetonitrile to prepare a stock solution at 10 mg/mL. HPLC grade acetonitrile (>99.9%) was also obtained from Sigma-Aldrich. HPLC grade water was prepared in house using Milli-Q Nanopure water purification system (Billerica, MA).

2.2 Instrumentation

The instrument used in this experiment was assembled primarily from components from the Agilent 1290 Infinity II line of UHPLC modules. This included: a high speed binary pump (p/n: G7120A) equipped with an Agilent Jetweaver V35 mixer, a 2D-LC switching valve (p/n: 5067-4214), and a DAD detector (G7117B) equipped with the ultra-low dispersion flow cell (G4212-60038). In some experiments, peaks were detected on-capillary by laser-induced fluorescence using a Picometrics Zetalif LIF detector (p/n: DIS-UV-01). The analog output signal of the LIF was recorded using a Universal Interface Box (Agilent, p/n: G1390B) set to acquire data at the maximum rate of 100 Hz. Analyte solutions were infused directly into the 2D-LC valve using a Hamilton Gastight 1000 µL injection syringe and a Harvard Apparatus syringe pump (p/n: 55-2226). The valve was fixed with two nominally identical stainless steel sample loops with volumes of about 2 µL (200 mm x 120 µm i.d.). The union (0.010” through-hole) and tee-piece (0.020” through-hole) shown in Fig. 2 were from Chromtech (Apple Valley, MN; p/n U-435 and U-428, respectively), and the inline filter was from Agilent (p/n: 5067-6189). The ‘prototype splitter pump’ shown in Fig. 2 was described previously [24] and used in this work to withdraw effluent from the flow path at a controlled rate. PEEK capillary tubing was from Chromtech and cut to length as needed for the different post-split tubing lengths and diameters indicated in Figs. 4A and 5. Stainless steel capillaries used for post-split tubing as shown in Figs. 4B, 6, and 7 were prototype parts prepared in-house by Agilent. LC columns used as described in Fig. 2 were from Agilent. Teflon-coated fused silica was from Polymicro (Phoenix, AZ). The Agilent instrument components were controlled using ChemStation software (Rev. C.01.07 SR3 [465]), and analyte peaks were integrated using the same software.

2.3 Methods

Measurements with UV Detection *(configurations $A_1/B_1/C_1$ in Fig. 2)*

A 100 µg/mL sample of acetophenone in 40/60 ACN/water was infused into the 2D-LC valve at a flow rate of 16 µL/min. The column was a 50 mm x 2.1 mm i.d. Zorbax SB-C18 (3.5 micron). The mobile phase was 50/50 ACN/water (k of acetophenone ~ 2), the column was not temperature controlled, and the flow rate through the column was 1.0 mL/min. The 2D-LC valve was switched once every 30 seconds such that eight injections were made over a four-minute period. Actual flow rates through the two outlet branches of the tee-piece were determined by measuring the time required to fill an empty barrel of a Gastight syringe using a stopwatch.

Measurements LIF Detection *(configurations B_2/C_2 in Fig. 2)*

Teflon-coated fused silica was used after the union shown in Figs. B2/C2 to enable on-capillary detection; the inner diameter was 50 µm, the total length was 150 mm, with 50 mm of capillary between the union and the on-capillary detection point. Assuming the length of the illuminated zone is 3 mm, the calculated volume of the illuminated effluent is about 6 nL. For these experiments the sample was 30 µg/mL fluorescein in 50/50 ACN/water. The column was a 30 mm x 2.1 mm i.d. Zorbax SB-C18 (3.5 micron). The mobile phase was 50/50 ACN/buffer, where the buffer was 20 mM ammonium acetate adjusted to pH 6.0 with formic acid (under these conditions the retention factor of fluorescein is about 0.6). The column was not temperature controlled, and the flow rate through the column was 1.0 mL/min. Actual flow rates through the two outlet branches of the tee-piece were determined by measuring the time required to fill an empty barrel of a Gastight syringe using a stopwatch.

Determination of Peak Standard Deviations

Peak widths were measured (in time units) at 4.4% height using the native integrator in Chemstation, and then divided by 5 to obtain $\sigma_{4.4}$ (in time units). These time-based standard deviations were then converted to volume units by multiplying by the experimentally determined flow rate through the detector cell (F_{det}).

3. Results and Discussion

The central argument of this paper depends on the idea that when the effluent exits the analytical column and is split into two flow paths, that mass (both analyte mass and effluent mass) is conserved. If this is true then:

1) The sum of the flow rates exiting the split point must be equal to the flow rate entering the split point; and
2) The ratio of the peak standard deviations in volume units after ($\sigma_{v,ps}$) and before ($\sigma_{v,col}$) the split is equal to the ratio of the flow rate after (F_{det}) and before (F_{col}) the split as follows:

$$\frac{\sigma_{v,ps}}{\sigma_{v,col}} = \frac{F_{\mathrm{det}}}{F_{col}} \tag{1}$$

Effect of Flow Splitting on Peaks Detected by UV Absorption

As an initial test of this framework we measured peak standard deviations with and without splitting under conditions where the tee-piece used for splitting, the post-split tubing, and the detector cell (~ 0.6 μL^2 [3]) are not expected to add significantly to the measured standard deviations. The legitimacy of the assumption that the tee-piece and post-split tubing do not add significantly to the measured variances without splitting is verified by the results shown in Fig. S1, as the difference in σ_v is less than 2% (6.66 µL vs. 6.54 µL). The results of the experiments involving different split ratios are shown in Fig. 3, where the split ratio was varied from 1:0 (i.e., no split) to 1:36 (i.e., 1 part of the flow to the detector, and 36 parts of the total flow to waste), but the post-split tubing was fixed at 100 mm x 120 µm i.d. PEEK. All chromatograms on the left are plotted on a time axis. For the split ratios 1:0, 1:1.6, and 1:4.7 the shift in the arrival time of the peak at the detector is hardly perceptible by eye. Even for the 1:36 split ratio the shift in retention time is modest at about 28%. This is because the transit time of the analyte between injector and detector is dominated by time spent in the analytical column. On the other hand, when the same peaks are plotted on a volume axis constructed by multiplying time and the flow rate measured at the outlet of the detector cell, we see that the peak volume obviously decreases as the split ratio increases. Although the analyte concentration at the peak apex should be independent of the split ratio, and thus the peak heights should all be the same, the peak observed with the 1:36 split ratio is shorter than expected because it is wider than expected due to dispersion in the post-split tubing and detector that is not negligible for a peak with this volume peak. The peak standard deviation measured with no split (i.e., 1:0; σ_v measured at 4.4% height) is about 6.7 µL, but with the 1:36 split it is just 0.57 µL. This result is entirely consistent with the idea that only a portion of the analyte mass entering the split point makes it to the detector, because some of the mass goes to waste, and thus the σ_v values measured at the detector must be smaller. This comparison makes clear the importance of viewing such results in volume terms.

If Eq. 1 is correct, then it should also be possible to predict the expected peak standard deviation after the split point ($\sigma_{v,ps}$) by rearrangement of Eq.1 if the peak standard deviation prior to the split point is known ($\sigma_{v,col}$ – measured using configurations B_1/B_2), and the flow rates out of the split point are known (F_{col} and F_{det}). That is:

$$\sigma_{v,ps} = \sigma_{v,col} * \frac{F_{\text{det}}}{F_{col}} \tag{2}$$

A comparison of $\sigma_{v,det}$ values (used here as a proxy for $\sigma_{\text{v,ps}}$) measured experimentally or estimated using Eq. 2 is shown in Fig. 4A. We see that the two values are very close for a split ratio of 1:1.6. The trend in the estimated values is consistent with the trend in the measured values, but the difference between the two values increases as the split ratio increases. On one hand we take the agreement between the trends as further evidence that Eq. 1 is consistent with what physically happens during the splitting process; on the other hand it is likely that the increasing differences between the values at higher split ratios is due to non-negligible contributions to the measured peak standard deviations from the post-split tubing and the detector cell itself when the split ratio is large. This is not unexpected, but in our view these initial experiments were nevertheless useful because they closely resemble “typical conditions” – that is, 120 µm i.d. post-split tubing and a low dispersion UV detector flow cell. A more accurate estimation of $\sigma_{v,det}$ is discussed further on in this discussion (see Eq. (5)).

Now, if the post-split peak standard deviation is dictated by Eq. 2, then we would expect that the peak standard deviation measured at the detector is influenced by the dimensions of the post-split tubing. In addition, the degree of increase in peak variance due to dispersion in the post-split tubing should increase as the split ratio increases. Figure 5 shows the results of a series of measurements where the dimensions of the post-split tubing and split ratios were changed, holding all other conditions of configuration C_1 constant. We see that when the post-split tubing is fixed the peak standard deviations measured at the detector ($\sigma_{v,det}$) decrease (Fig. 5A), as expected, with increasing split ratio, consistent with Fig. 4A. On the other hand, for a given fixed split ratio, the peak standard deviation measured at the detector increases as the volume of the post-split tubing increases. To better illustrate this, Figure 5B shows the percent increase in peak variance upon changing to larger post-split tubing, relative to the 100 mm x 120 µm i.d. post-split tubing (referred to hereafter as $\sigma^2_{v,\text{det},ref}$) used to obtain the results in Fig. 4A; this is calculated as $100\% * \left(\sigma^2_{v,\text{det}} - \sigma^2_{v,\text{det},ref}\right) / \sigma^2_{v,\text{det},ref}$. Here we see that the relative increase in the variance due to a change to a particular piece of post-split tubing does indeed increase as the split ratio increases because the denominator in the preceding equation decreases dramatically due to the increased split ratio. Moreover, this increase is much larger for longer pieces of post-split tubing.

Effect of Flow Splitting on Lower Volume Peaks – On-Capillary Fluorescence Detection

The conditions used for the experiments discussed above were chosen to produce peak variances entering the split point that would be relatively large (about 45 μL^2) compared to the expected variance due to the default post-split tubing (100 mm x 120 µm i.d.) and the low-dispersion DAD flow cell (about 0.6 μL^2 [3]). These conditions served us well in that they enabled an initial confirmation of our framework without using exotic measurement conditions. However, this level of post-column variance (~ 45 μL^2) of does not reflect the state-of-the-art in terms of narrow, low volume peaks that are produced under some LC and most 2D-LC conditions. For example, a 30 mm x 2.1 mm i.d. column has a dead volume of about 52 µL (assuming a total porosity of 50%). The expected peak volume (measured by σ) at the outlet of the column under gradient elution can be calculated using Eq. 3, where N is the plate number, V_m is the dead volume of the column, and k_e is the retention factor of the analyte at the point of elution.

$$\sigma_{v,col} = \frac{V_m}{\sqrt{N}}\left(1+k_e\right) \qquad \text{Eq. 3}$$

If we consider such a column with a plate number of 7,000, operated under gradient elution for the separation of peptides such that k_e is about 2, we find that the expected peak volume is 1.9 µL, and the expected variance 3.5 μL^2. Therefore, in a second set of experiments we chose conditions (30 mm x 2.1 mm i.d. column, $k_e \sim 0.6$) that would produce peaks entering the split point with variances of 6.7 μL^2 so that we could study the effects of split ratio and post-split tubing on the volumes of these smaller (i.e., lower volume) peaks, where their effects would be more pronounced.

Figure 4B shows results similar to those in Fig. 4A, but now using the instrument configurations B_2 and C_2 shown in Fig. 2, which involve a narrower post-split tube (100 mm x 50 µm i.d.) as a reference condition, and on-capillary laser-induced fluorescence (LIF) detection to drastically reduce the dispersion contribution from the detector. Without any splitting (1:0) the peak standard deviation measured at the LIF detector ($\sigma_{v,det}$) is about 2.5 µL. As the split ratio is increased, the measured peak standard deviation (black bars) decreases, consistent with the results shown in Fig. 4A. At the highest split ratio of 1:19 used in this set of experiments, the measured peak standard deviation is just 0.3 µL and the corresponding peak variance thus just below 0.1μL^2. The white bars show the peak standard deviations we expect to see in this case by simply applying Eq. 2. Again we see that the trend in measured peak standard deviations is similar to the trend in the estimated values, but in this case the agreement between the two sets of numbers is a bit better than in Fig. 4A, presumably because the post-split sources of dispersion have been reduced significantly.

What we are more interested in from a practical point of view is – what is the effect of post-split tubing on peak variances measured at the detector for different split ratios? The results shown in Fig. 6 begin to answer this question. Looking at Panel A we see several trends that are consistent with our intuitive expectations. First, when the length of the post-split tubing is fixed, but the diameter is increased, the standard deviations of the detected peaks increase for a given split ratio (i.e. comparison of the black, blue, and green bars). This makes sense because the peak dispersion in the post-split tubing is expected to increase with increasing tubing diameter (see Eq. 4, *vide infra*). Second, when the diameter of the post-split tubing is fixed, but the length is increased, we see that the standard deviations of the expected peaks increase as well. Again, this is expected because the dispersion of peaks in the post-split tubing is proportional to increasing tubing length (see Eq. 4). More interesting, though, is the increase in peak variance as the post-split tubing dimensions are changed, relative to the case with the narrowest and shortest tubing (100 mm x 50 µm i.d.), for different split ratios (Fig. 6, Panel B). Whereas the average increase in variance is just 13% when there is no split, this becomes much larger as the split ratio increases. In the case of the 1:19 split, what might seem like small changes in tubing dimensions can have a dramatic effect on peak variance. For example, changing the diameter of a 100-mm long post-split tube from 50 to 75 µm or 120µm increases the variance by 81 and 349% respectively. Keeping the narrow 50µm i.d., a four-fold increase in tubing length to 400 mm increases variance by 85% for the 1:19 split. These results are incredibly useful to experimentalists who need to decide how to set up a post-column flow splitting arrangement. Since for high split ratios the flow rate in the tubing towards the detector is low (e.g. 1/20th of the pump flow rate in the case of a 1:19 split), even very narrow 50 µm i.d. tubing does not generate a very high pressure drop, while avoiding excessive additional post-split band broadening. For example, a flow rate 0.1 mL/min through a 200 mm long 50 µm i.d., tubing, generates just 22 bar backpressure for a mobile phase with a viscosity of 0.001 Pa·s (i.e., the viscosity of water at 20 °C).

Considering the magnitude of the combined effect of choices about split ratio and post-split tubing dimensions on the observed peak variance at the detector naturally leads one to questions about whether or not these effects can be predicted. The results of such an exercise are shown in Fig. 7, which compares measured and predicted peak variances at the detector for four different split ratios and six different pieces of post-split tubing (24 total combinations). The predicted variances were calculated in two steps:

1) The expected post-split standard deviation at a given split ratio is calculated using the known post-split peak standard deviation without split, and the known flow rates into and out of the split point. Equation 2 is used here, as discussed above.

2) The expected dispersion in the post-split tubing ($\sigma^2_{v,ps-tub}$) is determined using Eq. 4 [5], where $L_{ps\text{-}tub}$ and $d_{ps\text{-}tub}$ are the length and diameter of the post-split tubing, D_m is the diffusion coefficient of the analyte in the mobile phase, and F_{det} is the flow rate in the post-split tubing.

$$\sigma^2_{v,ps-tub} = \frac{\pi^2 \cdot L^2_{ps-tub} \cdot d^4_{ps-tub}}{48 + 192 \cdot \pi \cdot L_{ps-tub} \cdot \frac{D_m}{F_{\mathrm{det}}}} \tag{4}$$

These values are then combined to calculate the predicted peak variance at the detector:

$$\sigma^2_{v,\mathrm{det}} = \left(\sigma_{v,col} * \frac{F_{\mathrm{det}}}{F_{col}} \right)^2 + \sigma^2_{v,ps-tub} \tag{5}$$

In Fig. 7 we again see that the predictions follow the experimentally determined values quite well for all investigated split ratios. These values are plotted on a single set of axes in Fig. S2. The median difference between experimental and predicted values across all of the conditions shown in Fig. 7 is -10%. Considering that these conditions result in a range of variances that vary by a factor of 235, we feel this level of predictive accuracy is quite good for such a simple model, and certainly good enough to guide practitioners in their choice of post-split tubing.

We have built a simple web-based tool to calculate the expected peak variance and standard deviation in the detector after a flow split that requires only the column parameters (dimensions, total porosity and plate count), retention at elution and diffusion coefficient of the considered compound, the split ratio and post column tubing dimensions (http://multidlc.org/flow_splitting). The tool also calculates the expected pressure drop originating from the post-split tubing. Example outcomes from these calculations are shown in Table 1 for different practically relevant scenarios involving columns of different dimensions, flow rates, split ratios, and post-split tubing. As one would expect, the larger volume columns are the least sensitive to dispersion in the post-split tubing. However, even for the large volume 50 mm x 4.6 mm i.d. column using the combination of a 1:10 split and 300 mm of 120 µm i.d. post-split tubing will result in a 57% loss in separation efficiency. In this case the problem is easily resolved by moving to a 75 µm i.d. post-split tubing which reduces the efficiency loss to 17%. When using smaller volume columns, however, the challenge is far more serious (e.g., see the 30 mm x 2.1 mm i.d. column results). Even when using a modest split of 1:2 and a 300 mm x 75 µm i.d. post-split tube the efficiency loss is 37%. In this case the loss can be managed by moving to a shorter (200 mm) and narrower (50 µm i.d.) tube, which reduces the loss to 6.5%.

4. Conclusions

In this work we have worked to describe a framework for thinking about potential peak dispersion that occurs in post-column capillaries in LC systems where flow splitting is used prior to detection. We have highlighted the point that when flow splitting is used, both the mobile phase flow rate and the analyte peak volume are split at the split point. This can lead to analyte peak volumes that are quite small ($\sigma << 1$ µL), such that the contribution of dispersion in connecting capillaries between the split point and detector to the observed peak variance can be strongly dependent on the dimensions of the capillary used. Predictions of the expected increase in peak width as a function of the capillary dimensions used post-split point, based on our own dispersion measurements described here, show that increases on the order of 100% are possible for small molecules even when using capillaries on the order of 100 mm x 120 µm i.d. As the performance of the LC column improves (i.e., peak width decreases) or the split ratio is increased, the expected increases in dispersion due to the post-split tubing become even more serious. Experiments involving flow splitting and different post-split capillaries have validated the trends predicted by the framework described here. One clear implication of this study, which should be easy to act on in practice, is that when flow splitting is used and there is only one detector used, the split point should be located as close as possible to the detector so that the distance traveled by the analyte band is as short as possible following the split. When two detectors are used the placement of the splitter will require a more careful consideration of the detectors and split ratio involved. Given the dependence of dispersion in capillaries on analyte molecular weight as described by other groups [25–27], future work should investigate the effect of flow splitting on dispersion for much larger molecules as well (e.g., proteins). It is expected (see Eq. (4)) that the dispersion from the post-split capillary will increase significantly with decreasing diffusion coefficient.

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References

[1] J. Sternberg, Extracolumn contributions to chromatographic band broadening, in: Adv. Chromatogr., Marcel Dekker, 1966: pp. 205–270.

[2] H.H. Lauer, G.P. Rozing, The selection of optimum conditions in HPLC I. The determination of external band spreading in LC instruments, Chromatographia. 14 (1981) 641–647. https://doi.org/10.1007/BF02291104.

[3] M.M. Dittmann, Chapter 3 - The Issue of External Band Broadening in HPLC/UHPLC Devices, in: S. Kromidas (Ed.), HPLC Expert II Find Optim. Benefits Your HPLCUHPLC, Wiley-VCH, Weinheim, 2017: pp. 73–99.

[4] M. Gilar, T.S. McDonald, F. Gritti, Impact of instrument and column parameters on high-throughput liquid chromatography performance, J. Chromatogr. A. 1523 (2017) 215–223. https://doi.org/10.1016/j.chroma.2017.07.035.

[5] K.J. Fountain, U.D. Neue, E.S. Grumbach, D.M. Diehl, Effects of extra-column band spreading, liquid chromatography system operating pressure, and column temperature on the performance of sub-2-µm porous particles, J. Chromatogr. A. 1216 (2009) 5979–5988. https://doi.org/10.1016/j.chroma.2009.06.044.

[6] K. Vanderlinden, K. Broeckhoven, Y. Vanderheyden, G. Desmet, Effect of pre- and post-column band broadening on the performance of high-speed chromatography columns under isocratic and gradient conditions, J. Chromatogr. A. 1442 (2016) 73–82. https://doi.org/10.1016/j.chroma.2016.03.016.

[7] G. Desmet, K. Broeckhoven, Extra-column band broadening effects in contemporary liquid chromatography: Causes and solutions, TrAC Trends Anal. Chem. 119 (2019) 115619. https://doi.org/10.1016/j.trac.2019.115619.

[8] S.R. Groskreutz, M.M. Swenson, L.B. Secor, D.R. Stoll, Selective comprehensive multi-dimensional separation for resolution enhancement in high performance liquid chromatography, Part II–Applications, J. Chromatogr. A. 1228 (2012) 41–50. https://doi.org/10.1016/j.chroma.2011.06.038.

[9] M. Sorensen, D.C. Harmes, D.R. Stoll, G.O. Staples, S. Fekete, D. Guillarme, A. Beck, Comparison of originator and biosimilar therapeutic monoclonal antibodies using comprehensive two-dimensional liquid chromatography coupled with time-of-flight mass spectrometry, MAbs. 8 (2016) 1224–1234. https://doi.org/10.1080/19420862.2016.1203497.

[10] D.R. Stoll, D.C. Harmes, G.O. Staples, O.G. Potter, C.T. Dammann, D. Guillarme, A. Beck, Development of Comprehensive Online Two-Dimensional Liquid Chromatography-Mass Spectrometry using Hydrophilic Interaction and Reversed-Phase Separations for Rapid and Deep Profiling of Therapeutic Antibodies, Anal. Chem. 90 (2018) 5923–5929. https://doi.org/10.1021/acs.analchem.8b00776.

[11] R.B. Cole, ed., Electrospray ionization mass spectrometry: fundamentals, instrumentation, and applications, Wiley, New York, 1997.

[12] M. Rodriguez-Aller, R. Gurny, J.-L. Veuthey, D. Guillarme, Coupling ultra high-pressure liquid chromatography with mass spectrometry: Constraints and possible applications, J. Chromatogr. A. 1292 (2013) 2–18. https://doi.org/10.1016/j.chroma.2012.09.061.

[13] K.M. Kalili, J. Vestner, M.A. Stander, A. de Villiers, Toward Unraveling Grape Tannin Composition: Application of Online Hydrophilic Interaction Chromatography × Reversed-Phase Liquid Chromatography–Time-of-Flight Mass Spectrometry for Grape Seed Analysis, Anal. Chem. 85 (2013) 9107–9115. https://doi.org/10.1021/ac401896r.

[14] K. Sandra, M. Steenbeke, I. Vandenheede, G. Vanhoenacker, P. Sandra, The versatility of heart-cutting and comprehensive two-dimensional liquid chromatography in monoclonal antibody clone selection, J. Chromatogr. A. 1523 (2017) 283–292. https://doi.org/10.1016/j.chroma.2017.06.052.

[15] M. Bernardin, F. Bessueille-Barbier, A. Le Masle, C.-P. Lienemann, S. Heinisch, Suitable interface for coupling liquid chromatography to inductively coupled plasma-mass spectrometry for the analysis of organic matrices. 1 Theoretical and experimental considerations on solute dispersion, J. Chromatogr. A. 1565 (2018) 68–80. https://doi.org/10.1016/j.chroma.2018.06.024.

[16] D. Spaggiari, S. Fekete, P.J. Eugster, J.-L. Veuthey, L. Geiser, S. Rudaz, D. Guillarme, Contribution of various types of liquid chromatography–mass spectrometry instruments to band broadening in fast analysis, J. Chromatogr. A. 1310 (2013) 45–55. https://doi.org/10.1016/j.chroma.2013.08.001.

[17] T. Hetzel, T. Teutenberg, C. Portner, J. Tuerk, Chapter 5 - Requirements of LC-Hardware for the Coupling of Different Mass Spectrometers, in: S. Kromidas (Ed.), HPLC Expert II Find Optim. Benefits Your HPLCUHPLC, Wiley-VCH, Weinheim, 2017: pp. 171–192.

[18] F. Gritti, T. McDonald, M. Gilar, Accurate measurement of dispersion data through short and narrow tubes used in very high-pressure liquid chromatography, J. Chromatogr. A. 1410 (2015) 118–128. https://doi.org/10.1016/j.chroma.2015.07.086.

[19] G. Taylor, Dispersion of Soluble Matter in Solvent Flowing Slowly through a Tube, Proc. R. Soc. Math. Phys. Eng. Sci. 219 (1953) 186–203. https://doi.org/10.1098/rspa.1953.0139.

[20] R. Aris, On the Dispersion of a Solute in a Fluid Flowing through a Tube, Proc. R. Soc. Math. Phys. Eng. Sci. 235 (1956) 67–77. https://doi.org/10.1098/rspa.1956.0065.

[21] J.G. Atwood, M.J.E. Golay, Dispersion of peaks by short straight open tubes in liquid chromatography systems, J. Chromatogr. A. 218 (1981) 97–122. https://doi.org/10.1016/S0021-9673(00)82050-6.

[22] M.J.E. Golay, J.G. Atwood, Early phases of the dispersion of a sample injected in poiseuille flow, J. Chromatogr. A. 186 (1979) 353–370. https://doi.org/10.1016/S0021-9673(00)95261-0.

[23] A. Shankar, A.M. Lenhoff, Dispersion in round tubes and its implications for extracolumn dispersion, J. Chromatogr. A. 556 (1991) 235–248. https://doi.org/10.1016/S0021-9673(01)96224-7.

[24] M.R. Filgueira, Y. Huang, K. Witt, C. Castells, P.W. Carr, Improving peak capacity in fast online comprehensive two-dimensional liquid chromatography with post-first-dimension flow splitting, Anal. Chem. 83 (2011) 9531–9539. https://doi.org/10.1021/ac202317m.

[25] A. Goyon, D. Guillarme, S. Fekete, The importance of system band broadening in modern size exclusion chromatography, J. Pharm. Biomed. Anal. 135 (2017) 50–60. https://doi.org/10.1016/j.jpba.2016.12.004.

[26] W.K. Marek, D. Sauer, A. Dürauer, A. Jungbauer, W. Piątkowski, D. Antos, Prediction tool for loading, isocratic elution, gradient elution and scaling up of ion exchange chromatography of proteins, J. Chromatogr. A. 1566 (2018) 89–101. https://doi.org/10.1016/j.chroma.2018.06.057.

[27] K. Baran, W.K. Marek, W. Piątkowski, D. Antos, Effect of flow behavior in extra-column volumes on the retention pattern of proteins in a small column, J. Chromatogr. A. 1598 (2019) 154–162. https://doi.org/10.1016/j.chroma.2019.03.060.

Figure Captions

Figure 1. Implications of the conservation of analyte mass on the standard deviations of peaks exiting the split point. The convention used in this paper is that a split ratio of X:Y means that the fraction X/(X+Y) of the peak goes to the detector, and the fraction Y/(X+Y) goes to waste.

Figure 2. Schematic representations of instrument setups used in this work. Configurations A have no T-split, B has a T-split with metal plug, and C has a T-split and a Prototype Flow Splitter to control the split ratio. Configurations with subscript 1 use a DAD detector and a Zorbax SB-C18 column (50 mm x 2.1 mm i.d.; 3.5 micron); those with subscript 2 use the same column chemistry, but with on-capillary LIF detector and a shorter column (30 mm x 2.1 mm i.d.; 3.5 µm). In configurations B_2 and C_2 the variable post-split tubing at the outlet of the split point is connected to the fused silica of the LIF by a zero dead volume union connector. All tubing dimensions indicated are length x i.d.

Figure 3. Effect of post-column flow splitting on peak characteristics. Each row shows the same chromatogram twice – once on a time axis (left), and once on a volume axis (right) calculated using the flow rate measured at the outlet of the detector cell (F_{det}). The split ratio used to obtain each peak is shown at the right of the row (portion to detector : portion to waste). Configuration C_1 from Fig. 2 was used with 100 mm x 120 µm i.d. PEEK tubing between the split point and the detector. Peaks in panels b, d, f, and h were aligned vertically to facilitate comparison on a single x-axis.

Figure 4. Comparison of experimental (error bars represent one standard deviation; n=8) and predicted (using Eq. 2) peak standard deviations for different split ratios. A) Experimental values measured using configuration B_1 or C_1, with the 50 mm x 2.1 mm i.d. column (analyte = acetophenone) and constant post-split tubing of 100 mm x 120 µm i.d.. B) Experimental values measured using configuration B_2 or C_2, with the 30 mm x 2.1 mm i.d. column (analyte = fluorescein) and constant post-split tubing of 100 mm x 50 µm i.d. Standard deviations in volume units are calculated using the flow rate measured at the outlet of the detector cell (F_{det}).

Figure 5. Effect of different dimensions of post-split tubing with different split ratios; measurements were made using configuration B_1 (no split; 1:0) or C_1 (split). A) Peak standard deviations measured at the detector (error bars represent one standard deviation, n=8); B) Percent increase in peak variance ($\sigma^2_{v,\det}$) relative to the variance obtained from the smallest post-split tube of 100 mm x 120 µm i.d.

Figure 6. Effect of different dimensions of post-split tubing with different split ratios; measurements were made using configuration B_2 (no split; 1:0) or C_2 (split). A) Peak standard deviations measured at the detector (error bars represent one standard deviation, n=8); B) Percent increase in peak variance ($\sigma^2_{v,\det}$) relative to the variance obtained from the smallest post-split tube of 100 mm x 50 µm i.d.

Figure 7. Comparison of experimental (error bars represent one standard deviation; n=8) and predicted (using Eq. 5, with $D_m = 1 \times 10^{-9}$ m^2/s) peak variances measured using configuration B_2 or C_2, with post-split tubing of different dimensions, and different split ratios. Variances in volume units are calculated using the flow rate measured at the outlet of the detector cell (F_{det}). All post-split tubing dimensions are length x i.d.