Extra-column Band Broadening in Ultra High Performance Liquid Chromatography

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EXTRA-COLUMN BAND BROADENING IN ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Advances in column technologies for high performance liquid chromatography (HPLC) have led to the use of small, highly efficient packing materials. The use of these materials requires short, small diameter columns as well as instruments capable of withstanding high pressures (up to 1000bar) and sometimes temperature (in excess of 100°C), a technique dubbed ultra-high performance liquid chromatography or UPLC. The advantage is a greater than ten-fold reduction in analysis time without a loss of peak capacity or resolution. Due to the small volumes inherent in the new columns, the extra-column volumes of the instrument can become a significant source of dispersion leading to extra-column broadening of chromatographic peaks. Uncontrolled or accounted for, this variance severely limits the separation potential of improved column packings and reduces the accuracy of evaluations of instruments and columns. An investigation is made of the source and nature of the band broadening in instrumental components with an eye towards reduction of variance without loss of performance. Different methods for calculating the degree of extra-column band broadening are discussed. Applications of the calculated data for evaluation of the kinetic parameters of UPLC are reviewed.

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1.0 INTRODUCTION

High performance liquid chromatography, or HPLC, has been an invaluable tool in a chemist's toolbox for almost 40 years. Until recently, the average commercially available column had an internal diameter of 4.6mm and was packed with porous particles 5µ in diameter. A typical run time was 20-40 minutes in order to obtain suitable resolution of analytes. In the past decade, however, advances have been made in developing smaller, thermally stable, or differently structured column packing materials. This has necessitated a push beyond the conventional instrument limits of 400bar of pressure with temperature maximums below 60°C, resulting in tremendous gains in analysis speed without a loss of resolution. A ten-fold reduction in run time can reasonably be achieved, meaning analysis times can be reduced to the two-minute range or less. Such short analysis times can be a benefit when screening large numbers of pharmaceutical samples, running validation tests, or monitoring complex biological reactions in real time.

The reduction in particle size leads to a reduction in column diameter and length, resulting in a greatly reduced column volume. The volumes of the extra-column components – the injector, the connection tubing before and after the column, and the detector - become large relative to the small column volume and are a more significant source of band-broadening than in a conventional HPLC system. Quantification of this contribution to band-broadening is critical to making an accurate assessment of the efficiency of a column or a chromatographic system. In order to obtain the maximum performance available from the new generation of

packing materials, these sources of band-broadening must be minimized. Studies have been performed focusing on these issues as interest in ultra-high pressure liquid chromatography (UHPLC) and high temperature liquid chromatography (HTLC) has grown.

1.1 BAND BROADENING OVERVIEW

A chromatographic peak is a representation of the distribution of concentration of the analyte over time; the shape of this peak is affected by each component of the chromatographic instrument.[1] Sources of extra-column band broadening are well known and have been studied extensively for conventional HPLC systems.

Statistical moments are used to describe the size, location, and shape of a peak. The zeroth moment gives the area under a curve. The first moment defines the mean of the distribution. The second central moment describes the variance of the distribution about the mean. Skewness, the degree of asymmetry of a peak, is related to the third statistical moment and is used to describe fronting and tailing.

The second central moment, σ^2, is a measure of the degree of band-broadening of a chromatographic peak. The total variance of an observed peak, assuming all contributions are independent, is accepted as being additive, as given by the equation:[1,2]

$$\sigma_{tot}^2 = \sigma_{col}^2 + \sigma_{inj}^2 + \sigma_{cap}^2 + \sigma_{det}^2 \qquad (1)$$

which indicates that the total variance is equal to the sum of the variance from the column, the injector, the connecting capillaries, and the detector, respectively. As the band of analyte molecules moves through the instrument, each component affects the distribution of concentrations in its own fashion. A chromatographic peak cannot pass through a component

completely unaffected, but the degree and nature of these effects determines the quality of the eluted peaks. Much attention is given to evaluating the band broadening that occurs inside the column. In HPLC with conventional columns, this is the primary source of peak broadening, so the extra-column contributions are seldom considered. When the extra-column band broadening becomes large relative to the column band broadening, as can happen in UPLC, there is a loss in the maximum obtainable efficiency of the column

The efficiency of a chromatographic separation is commonly referred to in terms of the number of theoretical plates (N). The higher the number of theoretical plates, the more efficient the separation. A simple expression for the number of plates is:

$$N = \frac{t_R^2}{\sigma^2} = \frac{t_R^2}{\left(\frac{W}{4}\right)^2} \qquad (2)$$

where t_R is the retention time, W is the peak width at the base, and σ is the standard deviation. For a Gaussian peak the standard deviation is approximately W/4. Therefore, the narrower the peak, the higher the number of theoretical plates and the more efficient the separation.

Instrumental components are, in part, composed of unpacked capillaries. The most obvious of these being the connections between the injector and column, and the column to the detector, but open capillaries also make up portions of the injection apparatus and detection cells as well. The behavior of a band of analyte in an open capillary under laminar flow conditions has been thoroughly analyzed. Convection moves the analyte molecules in the axial direction. In the absence of any radial or axial molecular diffusion, the band will become parabolic in shape due to the velocity gradients found between the center of the capillary and the walls. Molecules at the center of the capillary will have a higher velocity than those at the walls due to

friction between the mobile phase and the walls. The eluted peak will not be a Gaussian distribution.

Any diffusion of the molecules in the axial direction broadens the band. Radial diffusion, however, allows the analyte molecules to sample different velocities within the velocity gradient. This averages out the velocities of the individual molecules. Conditions allowing for sufficient radial diffusion result in a Gaussian distribution of the analyte band. When radial diffusion is significantly greater than axial diffusion, and the residence time in the capillary is sufficient for the analyte molecules to experience a range of velocities, the flow is said to be in the Taylor regime. The concentration band can be described by the effective diffusion coefficient:

$$D_{eff} = \frac{U^2 r^2}{48D} \tag{3}$$

Where *U* represents the mean linear velocity, *r* is the radius of the tube, and D is the molecular diffusion coefficient of the analyte. If both radial and axial diffusion affect the dispersion, the system is described as being in the Taylor-Aris regime, and effective diffusion coefficient is described by:

$$D_{eff} = D + \frac{U^2 r^2}{48D} \tag{4}$$

These two regimes are not separated by a sharp delineation, but depend on the ratio of the product of the mean velocity and radius to the molecular diffusion constant, a ratio known as the Peclet number (Pe):

$$Pe = \frac{Ur}{D} \tag{5}$$

When the following inequality, where L is the length of the capillary, is satisfied,

$$Pe \ll \frac{L}{r} \qquad (6)$$

radial diffusion is fast compared to axial convection. Generally speaking, for Pe > 70 the system is in the Taylor regime.[3]

In some cases the analyte band passes through the tube so quickly that there is insufficient time for either radial or axial diffusion to occur, and the motion is governed solely by convection. Plate height theory can be applied to open capillaries as well as to packed columns. For any capillary, there exists an optimum flow velocity wherein the height of a theoretical plate is at a minimum, maximizing the number of theoretical plates for the capillary. Velocities used in liquid chromatography are frequently several orders of magnitude larger than this optimum flow velocity, increasing the plate height and reducing the number of plates of the capillary. The complex peak shapes arising from dispersion of analytes in capillaries having less than 30 theoretical plates were modeled by Atwood and Golay.[4] In capillaries with a high number of plates in which long tube conditions apply, the volume normalized variance becomes asymptotic to the inverse square root of the normalized length. However, below three theoretical plates, the band broadening is significantly smaller than what would be predicted by long tube theory. Care must be taken to establish which conditions are most accurate when determining the band-broadening contribution of an open capillary.

As a general rule, the total loss of efficiency attributed to extra-column band broadening should only be 10% of the maximum efficiency of the column.[5] The extra-column variance of a typical HPLC instrument is estimated to be in the range of 75μL^2. If the total variance of a poorly-retained peak in a conventional column is 1500μL^2, this extra-column contribution represents only 5% of the column variance and is acceptable.[4] However, recent advances in

column packing materials have given rise to sub-2μm particles which theory indicates will give smaller plate heights:

$$N \, \alpha \, \frac{L}{d_p} \qquad (7)$$

In a packed column, the number of plates for a column is directly proportional to the length of the column (L) divided by the diameter of the particles (d_p). However, these particles generate significant back pressure in accordance with Darcy's law:

$$\Delta P = \varphi \ \frac{\eta \, Lu}{d_p^2} \qquad (8)$$

This shows that as particle size decreases, for comparable column length, mobile phase velocity (u) and viscosity (η), and flow resistance (φ), the pressure drop across the column increases. It was originally postulated that the back-pressure generated by small particles would be the primary limitation in column efficiency. While this is still true, advances in instrumental design have increased the achievable pressure from 400bar to 1000bar. To reduce the back-pressure to practical levels it is necessary to shorten the column length, and/or reduce mobile phase velocity and viscosity. Alternately, the maximum allowable pressure limit of the instrument can be raised by designing pumps and components that can withstand pressures higher than 400bar. In addition, friction resulting from the flow of the mobile phase through the small particle packings creates significant radial heating gradients within the column that can degrade chromatographic performance.[5,7] Reducing the column inner diameter minimizes these heat gradients. The net effect of these changes is a highly efficient column with a greatly reduced internal volume.

Considering that 5cm long columns with an inner diameter of 2.1mm packed with sub-2µm core-shell particles may give a peak variance as low as $2\mu L^2$ for compounds with retention factor k=1,[8] it becomes obvious that conventional instrumentation with $75\mu L^2$ extra-column variance is not capable of delivering the performance required to achieve the maximum benefit from new column technology.

2.0 INSTRUMENT COMPONENTS

We begin an exploration of the sources of extra-column band broadening by taking a closer look at the primary instrumental components responsible. Each instrument requires an injector to deliver the sample into the stream of mobile phase, connecting capillary tubing to connect the pump and injector to the column, a second tube to carry the eluent from the column to the detector, and a detector to generate and collect the resulting signal.

2.1 INJECTORS

An ideal injector will introduce a square pulse of analyte into the mobile phase with a minimal amount of tailing or asymmetry. Realistically there is always some perturbation of the pulse due to the physical characteristics of the injector and the means of delivery. The volume of the injector should be minimized so as to minimize the amount of mixing within the injector components, and abrupt diameter changes within the tubing should be minimized.[1] Injection volumes for UPLC should be minimized as well. They are typically in the range of 1µL for 1mm diameter columns, as the total system volume is small.

Although injection variance will change based on the size and shape of the sample plug, the assumed contribution to the variance from the injector can be estimated by the equation:

$$\sigma_{inj}^2 = \frac{V_{inj}^2}{12^2} \quad (9)$$

with V_{inj} being the injector volume.[2]

One means of achieving reduced injection volumes is to use a static-split method. Advantages of this method are the easy tuning of injection volumes and wide range of pressures that can be withstood, up to 1000bar.[7,9] The method can be wasteful of sample, which is a concern when the available amount of analyte is critical. Unfortunately it is also significantly less robust in terms of reproducibility of the amount injected.[5] Sample waste might be a concern in limited cases, but the lack of reproducibility severely limits this injection method for wide applicability. The gains in terms of reduced injection volume do not justify the loss of precision inherent in this method.

Prüß *et al*.[2] investigated a variety of injection modes using micro-valves with 150µm and 250µm stator bores and 10µL and 1µL sample loops to inject sample volumes from 0.1 to 1.0µL.

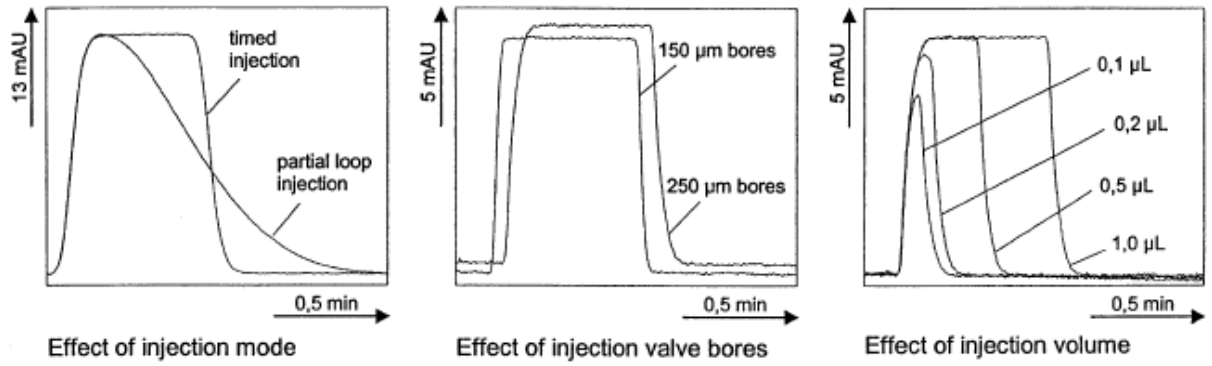

Figure 1: Effect of injection mode, valve bore size, and injection volume as determined by Prüß *et al*

Reprinted from Journal of Chromatography A, Vol 1016, Anja Prüß, Christine Kempter, Jens Gysler and Thomas Jira, Extracolumn band broadening in capillary liquid chromatography, 129-141. Copyright 2003, with permission from Elsevier.

When comparing partial loop injection, where the volume injected is determined by the amount dispensed from the syringe, to timed injection, where the volume injected is determined by the time the injection valve is left in the "inject" position and the flow rate, they found no difference in the front boundary of the sample, but a strong influence in the rear boundaries. This can be explained by understanding the path the sample travels in the two injection modes. In partial loop injection, the last portion of sample drawn remains in the stator bore and is minimally dispersed. This portion of the sample enters the injector first, forming the front boundary of the peak. The last portion injected into the chromatographic system has to pass through the stator bore for a second time, and thus experiences higher levels of dispersion. This last portion is cut off in timed injection, giving enhanced injection performance.

The distortion was reduced when stator bore inner diameters were reduced. The Prüß study suggests that stator bores are the sole source of dispersion in the injector, and that the inner diameter of the injection loop has no effect on the variance of the delivered pulse. In

contrast, studies done by Vissers *et al.*[10] on the effect of injection loop inner diameter on peak asymmetry and reproducibility found that the optimum inner diameter for injection loops of similar volume is 100µm. Holding total injector capillary volume and mobile phase velocity constant, with inner diameters above 150µm there was an increase in band broadening and a corresponding loss of reproducibility. Vissers suggested that larger inner diameter sample loops can act as a mixing chamber, reducing the reproducibility of the injections, while smaller inner diameter loops impeded the aspiration of the sample through the injector. The primary difference between the two results is in the Prüß study, timed injection mode was used when observing the effect of injector loop inner diameters, but in the work done by Vissers *et al*, partial loop injection was used. An assumption can be made that the improvements in variance observed using a timed-injection mode eliminated the effects of the varying inner diameters. Returning to the theory of dispersion in open capillaries, we know that for "short" capillaries with high flow velocities, the peak shape will not be Gaussian and exhibit a high degree of tailing. It is this portion of the peak that is cut off by the timed injection mode, explaining the seemingly contrary results. Timed injection appears to be the superior mode for reducing variance, as supported by both studies' results.

A comparison[8] of two different commercially available UPLC instruments, Waters' Acquity and Agilent's 1290 Infinity, was performed by Gritti and Guiochon to observe the efficiency of their injectors. The Acquity instrument's injection system has no needle seat capillary – the 5µL sample loop connects directly to the injection switching valve. In the 1290 Infinity injector, the sample is first drawn into a 20µL loop and flushed backward into the switching valve, though a needle and the needle seat capillary tube.

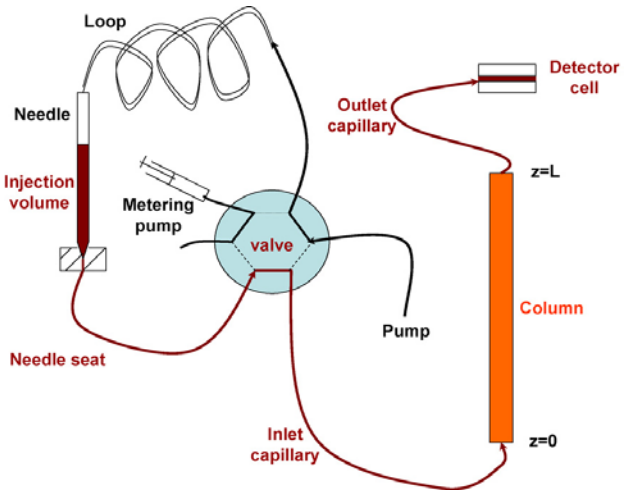

Figure 2: Schematic of the injection system of the Agilent 1290 Infinity system. Reprinted from Journal of Chromatography A, Vol 1217, Fabrice Gritti and Georges Guiochon, On the extra-column band-broadening contributions of modern, very high pressure liquid chromatographs using 2.1mm I.D. columns packed with sub-2µm particles, 7767-7689. Copyright 2010, with permission from Elsevier.

While the total volume of the injector parts in the Acquity is 8.7µL and the 1290 Infinity injector volume is 12.0µL, the contribution from extra-column variance for a 0.1 µL and 1.0µL sample was 4x and 2.5x larger, respectively, with the 1290 Infinity system than with the Acquity. This increase in variance is far above what would be expected based on the difference in volume alone. In an attempt to improve performance, the Infinity's needle seat capillary volume was reduced from 1.2µL to 0.9µL in the study to minimize variation from the injector.

While there is room for improvement in the volume of the Infinity injector, the injection volume has more of an impact in the Acquity system, and less impact on the 1290 Infinity. When injecting 1µL versus 0.1µL, peak variances were 6.9µL^2 compared to of 3.9µL^2. Most of the sample dispersion for the Acquity injector was assumed to take place in the injection loop before the sample enters the injector valve. This would confirm the advisability of keeping the inner diameter of the injection loop to a minimum. While the Infinity injector has higher off-the-shelf injector variance, the injector can be modified to reduce overall variance. Reproducibility

over a range of injection volumes is an asset in an analytical instrument. It would be interesting to observe if a change in injection loops minimizes or eliminates the variation with regard to injection volume. The comparison study also used partial loop injection, which can increase variance from the injector as established above.

Focusing the sample at the head of the column can be performed to reduce the effects of components prior to the column. Often the analyte is injected in a large volume of non-eluting solvent.[10] Gritti *et al* had success with a modified focusing technique wherein a calculated volume of weak solvent, typically water, is injected directly after the analyte.[11] The slug follows the analyte band through the instrumental components. When the analyte band reaches the head of the column it is overtaken by the weak solvent, significantly narrowing the band of analyte. After the weak solvent passes, the remainder of the elution is performed with the initial isocratic mobile phase. This focusing was shown to reduce a majority of the extra-column contributions to band broadening that occur prior to the column when a sufficient volume of weak solvent was used. On a practical level the method is less than desirable for a wide range of analytes because extremely hydrophobic compounds could precipitate in the system. It also introduces a potential source of variability because of the necessity to inject the weaker solvent manually. To use such a method on a practical scale the nature of the analyte would have to be considered to determine the optimum weak solvent composition and volume, making this mode of focusing interesting for limited situations but of little value in the broad sense. Many samples of interest, for example, are already very dilute in an aqueous matrix. The potential benefits of reduced variance may frequently be outweighed by the outlined difficulties. The technique has the greatest impact for compounds that have low retention factors, as the effect of extra-column band broadening is lesser for molecules having a high retention factor.[11] A similar advantage is

realized automatically when using gradient elution.[12] Extra-column band broadening has its greatest impact on isocratic elutions because the retention factor of the analytes can be tuned by the changes in mobile phase composition in gradient elution.

Temperature also impacts the performance of an injector. When designing high temperature HPLC systems, preheating of the mobile phase prior to introduction to the column is strongly recommended.[13] In one study[14] comparing the Acquity UPLC instrument to an Accela, made by Thermo Scientific, experimental values of external variance were consistently higher than theory for the Accela instrument, and a significantly higher variance was noted at 90°C compared to 30°C. It was suggested, but not proven, that the injection valve, which was contained within the column oven, may have been the source of the higher than expected variance. The authors suggest that the increase in temperature could result in higher dispersion at the time of injection at high temperatures. It is unclear if they are suggesting that the dispersion of the sample into the connecting capillary would be more affected, or that the sample dispersion within the injector would be higher. Regardless, a major limitation of the design is the temperature limitation introduced by including the injector in the oven compartment – operating temperatures above 70°-80°C are not recommended by the manufacturer.

2.2 CONNECTION TUBINGS

Generally speaking, variance in capillary tubes is directly proportional to the volume of the capillary. The most readily accessible means of minimizing band broadening is to reduce the diameter and length of the capillaries that connect the injector to the column, and the column to the detector. Diffusion in long, narrow tubes is frequently described using the Taylor equation:[2]

$$\sigma^2_{v,tubing} = \frac{\pi L r^4 u}{384 D} \quad (10)$$

where we can see that the variance due to the tubing increases with tubing length (L) and radius (r) as well as the mobile phase velocity (*u*) and is inversely proportional to the molecular diffusion coefficient (D). However, for capillaries used in UPLC, this model may be inaccurate. As previously discussed, in capillaries with fewer than 30 theoretical plates there is insufficient radial diffusion to relax the velocity gradients and create a Gaussian distribution. Once again, there is not a sharp delineation between the cases of insufficient radial diffusion and pure Taylor-Aris distributed concentration.

In an attempt to address the region between complete lack of diffusion and effective diffusion, Fountain *et al* developed a model to predict capillary band-broadening based on a random-walk computer simulation, described by the equation:[15]

$$\sigma^2_{v,tubing} = \frac{(\pi r^2 L)^2}{3 + 24\pi L \frac{D}{u}} \quad (11)$$

The model was then tested against a series of capillaries of the same length but varying inner diameters. Experimentally, extra-column variance was not observed to increase linearly with increasing mobile phase velocity as the Taylor-Aris model would predict. In the study it was found that for 50cm tubes varying inner diameters of 0.064mm to 0.254mm at low mobile phase velocities (<0.2mL/min) both Taylor-Aris and the random-walk models were accurate. At velocities above 0.2mL/min, band spreading was *lower* than that predicted by either the Taylor-Aris model or the random-walk derived model.

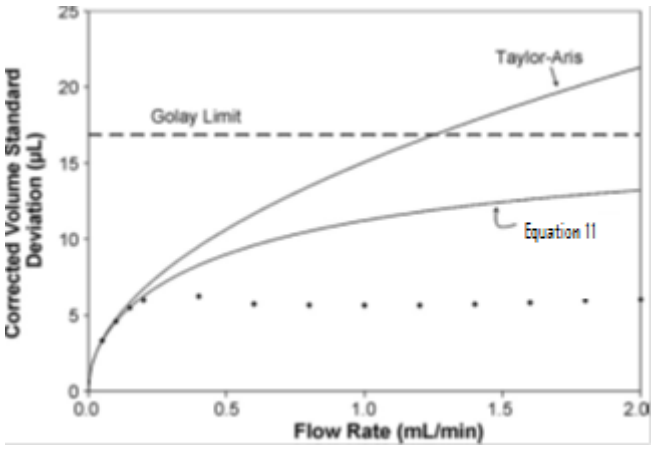

Figure 3: Taylor-Aris prediction compared to random-walk derived model "Equation 11", with experimental data indicated by dotted line. The Golay limit is a band spreading boundary determined by the volume of the tubing. Reprinted from Journal of Chromatography A, Vol 1216, Kenneth J. Fountain, Uwe D. Neue, Eric S. Grumbach and Diane 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, 5979-5988. Copyright 2009, with permission from Elsevier.

This result not surprising based on the work of Atwood and Golay.[4] The case of no diffusion of the analytes was predicated upon the actual mobile phase velocity (U) being significantly larger than the optimum mobile phase velocity (U_{opt}) calculated for the capillary. In their estimation a difference of $U/U_{opt} \geq 30$ was sufficient to lower the number of plates to the point where there was no time for diffusion. For their models they chose $U/U_{opt} = 100$. As flow velocities increase, the number of plates will continue to decrease. Atwood and Golay found that the volume-normalized variance for a capillary with 0.1 theoretical plates was one-fourth the variance predicted by long-tube theory. At flow rates above 0.2mL/min the experimentally determined band broadening appears to follow the model of no radial or axial diffusion. While it is true that the volume of the connection capillaries should be minimized to reduce external variance, the Taylor regime theory that mobile phase velocity is directly proportional to connection capillary variance does not appear to apply at all mobile phase velocities for the capillaries used in UPLC.

Gritti and Guiochon[8] went a step further and considered solvent viscosity effects in their investigation of the extra-column band broadening for naptho[2,3-a]pyrene eluted with acetonitrile and for 4-tert-butylphenol eluted with 65/35 methanol/water in the Acquity and 1290 Infinity systems. The connecting tubing on both instruments is roughly the same with a length of 75cm and inner radii of 65×10^{-4}cm. At very low flow rates (<0.2mL/min) the band broadening was three times greater for the aqueous methanol mobile phase than for pure acetonitrile. However, at flow rates above 0.2ml/min, only a 15% - 45% increase in band broadening was observed for the water/methanol solution compared to acetonitrile. The reason for this can be illustrated by revisiting the variance in an open capillary for the Taylor Aris regime:[8]

$$\sigma^2 = \frac{2DL}{u} + \frac{r^2 uL}{24D} \qquad (12)$$

The first term on the right-hand side describes axial diffusion, while the second term relates to radial diffusion. In the Taylor regime (Equation 10), the first term is negligible relative to the second term, and the variance is inversely proportional to the molecular diffusivity of the analyte. The molecular diffusivity decreases with increasing viscosity of the mobile phase. At velocities below 0.2mL/min, Taylor theory applies. Above 0.2mL/min the flow no longer falls neatly under this category and the effect of viscosity on variance is reduced. For an accurate analysis of the performance of an instrument for a given analyte, the actual flow rate and solvent composition to be used in analysis should be incorporated when calculating the degree of extra-column band broadening, bearing in mind that the dispersion models within the capillaries may not be consistent over the range of interest.

In an attempt to improve the performance of the Acquity instrument, the 127µm inner diameter heat exchanger tube, 550mm in length, was replaced with a "Viper" connecting tube from Dionex.[8] The replacement tube was 25cm long with an inner diameter of 130µm. This

reduced the extra-column variance from 3.9 to 2.2μL^2, demonstrating that there is room for improvement in this commercially available instrument by reducing the diameter and total volume of the connecting capillaries. The benefits of this exchange would have to be weighed against the loss of thermal stability afforded by the longer stabilizer capillary. For high temperature analysis the gain afforded by reduced variance may be negated by the reduction in temperature control.

Many chromatographers suggest that connectors for components be carefully selected to minimize abrupt changes in diameter leading to poorly swept voids which can increase mixing.[6] However in Prüß's study, zero-dead-volume unions of 150 and 250μm bore were used to connect a cut capillary of unspecified diameter with no significant increase to the band broadening.[2] Considering the previously addressed impact of the stator bores in the injector design, it is hard to believe that unions that introduce such an interruption in flow would not affect the band broadening. The largest capillary referenced in the study had an inner diameter of 150μm. The only truly zero volume unions are those where the cut ends of the tubing butt together perfectly, a precision that is difficult to achieve. In such a case the union does not actually make up any portion of the flow path. Zero-dead-volume unions are designed so that there are no unswept voids in the flow path.[16] This is only possible if the inner diameter of the connector matches that of the capillaries. To be fair, the referenced paper did not suggest that there was no additional band broadening due to the connections, but that the broadening was deemed insignificant. If the overarching goal of instrumental design is to minimize extra-column band broadening, it behooves the user to attempt to minimize all sources of broadening, not just the largest ones. There is nothing to be gained by using inappropriate capillary connections.

The downside of the reduction in tubing radius is the increase in back pressure, as described by the Poiseuille-Hagen equation:[17]

$$\Delta P_{ext} = \frac{8\eta L u}{\pi r^4} \qquad (13)$$

where η is the mobile phase viscosity, u is the volumetric flow rate, and *r* is the radius of the tube. A small reduction in radius increases the back pressure by an exponential factor of four. Columns packed with small particle packings generate considerable back pressure themselves, so it is clear that there is a limit to the reduction in inner diameter of the connecting tubings, even when using pumps capable of generating 1000bar of pressure. Extremely narrow tubings are also susceptible to clogging by particulates.[6] Because increasing the back pressure before and after the column reduces the available pressure drop across the column, it was feared that this pressure drop would negatively impact the quality and speed of the separation. DeCliq *et al.*[17] used kinetic plot analysis to observe whether there was a loss in performance in the presence of an extra-column pressure drop. Plots were prepared using pressure as a variable that changed with mobile phase velocity rather than assigning a fixed pressure maximum. The external pressure drop was estimated using the Poiseuille-Hagen equation and subtracted from the maximally available pressure from the instrument, in this case 400bar. Plots obtained under these constraints were compared to ones generated with an assigned maximum pressure. The effect of the reduced available column pressure on obtainable efficiency was observed to be negligible when using 3µm particle columns. The group had previously established that under high velocity conditions, for particle diameters 3µm or larger where the required number of plates for separation is lower than the optimum number of plates achievable, separation only weakly depends on available pressure.[18] This was not established for smaller packing particles, however. A study using kinetic plots to evaluate the impact of very small capillary radii on

smaller particle sizes with an upper pressure limit of 1000bar would be instructive to determine what the absolute limit is in terms of capillary back pressure.

Due to the relationship between pressure drop and viscosity, raising the temperature of the mobile phase can serve the dual purposes of reducing back pressure from the extra-column tubings and column as well as decreasing analysis times. A variety of heating methods, ranging from electrical heating of only the column to oven heating of the entire HPLC apparatus have been used to achieve high temperatures. It has been solidly established that to achieve the maximum benefit of elevated temperature, the mobile phase must be preheated prior to entering the column to avoid unacceptable peak broadening.[19] This is most often achieved by heating the connecting capillaries after the injector.[13,17, 20-22] To obtain the desired degree of heating, a given volume of solvent must travel through the heater with a residence time great enough to heat the solvent to the target temperature. The heater is typically incorporated into the connecting capillary between the injector and the column. The total volume of standard, non-heated connection capillaries are generally kept to a minimum. However, volume must be increased when using the capillaries as a means of heating. This volume increase may contribute an undesirable degree of band broadening.[20] One way to minimize the amount of tubing needed to raise the temperature of the mobile phase is to use a maximally efficient heating method. Water or oil baths have been demonstrated more effective at heat transfer compared to air baths.[23] Some researchers have found it advantageous to also heat the tubing connecting the pump to the injector, as well as the injector unit in addition to the connecting capillary to the column.[19, 24] This was accomplished by having a flow of preheated mobile phase converge with the injected sample at a very low volume "T".[19] While this diluted the sample somewhat, it significantly reduced the amount of tubing necessary after the injector. As previously discussed in section

2.1, heating prior to the injector may negatively impact the variance from the injector. The decrease in capillary variance would have to be verified as offsetting any impact of increased injector variance. Assuming increased injector variance was negligible or non-existent, however, this method appears to have the greatest benefit in terms of allowing for maximum pre-heating while minimizing the capillary volumes after the injector.

The studies performed by Thompson *et al.*[21] on thermal mismatch broadening establish that narrow bore columns gave better thermal equilibrium between the eluent and the column, though they still suggest that the necessary heating tubing is too long to make heating via air bath practical. For the limited case of very small column diameters, the requirements for heating aren't as difficult to overcome. Guillarme's group found that air baths gave acceptable performance up to 200°C when using microcolumns of 1mm inner diameter or less.[13] A low thermal mass method employed by Gu *et al.*[25] used an apparatus with resistive wire heating to achieve fine temperature control of a 250µm i.d. capillary column. Because of the nature of the rapid and tunable heating across the capillary column, the low mass of the capillary column, and the low flow rates used, it was unnecessary to preheat the mobile phase. Extra-column volume in the system was estimated at 1.1µL.

While running analyses at higher temperatures reduces somewhat the Taylor dispersion in the connecting capillaries as the increased molecular diffusion coefficient reduces the time required for radial diffusion, this is a small advantage, particularly considering that Taylor dispersion conditions do not rigorously apply to the capillaries. At the same time, peaks eluted at a higher temperature are narrower due to the reduction in plate height that occurs in the column at higher temperature. Narrower peaks are more affected by the extra-column band broadening.[17] Other experimental data[19] has shown that as temperature is increased, column

plate height decreases in the high-velocity region, but worsens at low velocity. The van Deemter equation:

$$H = A + \frac{B}{u} + Cu \qquad (14)$$

gives a suitable framework to understand this phenomenon. Plate height in the column is equal to the sum of the three terms: A, relating to eddy dispersion, B, arising from axial diffusion, and the C term accounting for the resistance to mass transfer in the stagnant mobile phase and stationary phase. A four-fold to nine-fold decrease in the C term was observed when incrementally increasing temperature from 25°C to 150°C. At the same time, the B term increases with increasing temperature.[15] While a decrease in retention factor is observed with increasing temperature, the molecular diffusion coefficient increases significantly. The C term is inversely proportional to the diffusion coefficient. Maintaining a low flow velocity while increasing temperature gives a net increase in plate height due to the increase in axial diffusion. At high velocity and high temperature, the C term is reduced by the increased molecular diffusion and the effect of axial diffusion is minimized by the high velocity. The conclusion that can be drawn is that to run at high temperature, velocity needs to be sufficiently high to avoid a loss in resolution as well as minimize the impact of extra-column band broadening on narrow peaks. Fountain *et al.* found that instrument-related band spreading decreased slightly with increasing temperature over a range of 30° - 90°C, a decrease attributed to the increase in the molecular diffusion coefficient.[15]

2.3 DETECTION

A variety of detection methods and devices are used for liquid chromatography, with the most popular being UV detection. Mass spectrometry is also widely used. Recent advances in column technology have also given rise to the use of monolithic columns to achieve rapid separation of analytes. Unfortunately the high mobile phase velocities frequently required for these columns make coupling with mass spectrometry difficult due to the large volume of solvent delivered with respect to time, while the low volumes and narrow peaks of UPLC are more suited to this detection method. Evaporative light scattering detection has also been investigated and found to be effective, but only for analytes with retention factors greater than five due to the additional band broadening afforded by the instrument, and using columns greater than 1mm due to the mass sensitivity of the device.[26]

Typical UPLC instruments are equipped with UV-Vis detectors. One of the challenges of the smaller volumes resulting from UPLC is the design of the detection cell. Smaller volumes of injected analyte result in smaller amounts of analyte to detect. Path lengths must be long enough to result in suitable signal strength for the detector, but not so long that the rapidly eluted and narrow peaks are broadened unnecessarily. Perpendicular flow cells minimize band broadening, but suffer from a loss of sensitivity due to short path lengths. Longitudinal flow cells give heightened sensitivity but suffer from excessive band broadening. Waters' UPLC low-volume detector cell uses Teflon AF to improve transmission efficiency in a 10mm longitudinal flow cell by maximizing internal reflectance of the light along the path of the cell.[27] This would serve to maximize the amount of light passing through the signal, but does not address the limitations of a longitudinal flow cell in terms of band broadening. One proposed improvement would be to use extended light path "bubble" cells, which are used in capillary electrophoresis.[2] This would

increase path length with a minimal increase in dispersion. Band broadening from UV detector cells appears to be less significant compared to that arising from the injector or capillary tubings, and is minimally addressed in the literature compared to other sources.

In addition to considering the physics of the flow cell, the data acquisition rate must be appropriate to the narrow, rapid peaks that are obtained with UPLC. In the study done by Gritti *et al*.,[6] the fastest flow rate was 4.7mL/min. Based on the calculated volume of their flow cell, the volume of the cell was replaced every 0.1 seconds. By keeping the data acquisition rate at 25Hz they ensured that the acquisition rate was always faster than the cell renewal rate. For lower flow rates, the acquisition rate was reduced to avoid unnecessarily large data files, though file size for storage is less of a concern than it was at the advent of computerized data collection. They then compared this data collection rate with the width of a peak recorded without a column in place. Because the width of a Gaussian peak is 4σ, they were assured to obtain a minimum of six data points per standard deviation, which should adequately describe a peak.

They also demonstrated that the values of the moments of extra-column band profiles are independent of the data acquisition rate as long as there are more than ten data points per peak. This was accomplished by varying the sampling rate of a band profile and randomly shifting the start and stop cutoffs times in the integration. By deleting a portion of the collected points and observing the effect on the calculated moment, they were able to observe that the data collection rate was adequate. Modern data collection software and computational storage space has made what was previously a limitation of concern for adequate data analysis into one that is facile to address.

3.0 QUANTIFICATION

Having observed the source of the extra-column band broadening contributions, we need a method of quantifying these variances. Once quantified, they can be used in evaluating the quality of a chromatographic system or column.

3.1 DETERMINING DEGREE OF VARIANCE

The standard accepted method of quantifying the extra column band broadening experimentally is by removing the column and replacing it with a zero dead volume union, and analyzing the profile of a non-retained peak. Determining how the measurements are to be taken is not straightforward, as peaks eluted without a column are prone to tailing and fronting significantly, which will greatly affect the quality of the calculated data. The simplest method is to take the width of the peak at some established height, divide by four, and square it. When analyzing the base of peaks, baseline noise can make it difficult to accurately establish the base of the peak. Gritti[4] stopped the integration of all peak profiles and measured peak width at 0.5% of the maximum height of the peak to eliminate the signal noise at the base of the peaks. More commonly the peak is measured at 10%, 13.4%, or 50% of total height. When comparing the extra-column variance of the Acquity and 1290 Infinity instruments,[8] Gritti and Guiochon accepted the tailing as a source of error in the extra-column contribution measurements because

the degree of tailing and fronting was similar in both instruments and would affect each estimate similarly. However, when studying column efficiency, it was suggested that the equation:[8]

$$\sigma^2 = u^2 \frac{(t_{1/2}^r - t_{1/2}^f)^2}{5.545} \qquad (15)$$

where u is the mobile phase velocity and $t^r_{1/2}$ and $t^f_{1/2}$ are the elution times of the rear and front parts of the peak at half-height, gives more accurate values because it minimizes the consequences of the tailing of the bands. This equation assumes a Gaussian peak shape, and therefore gives more accurate numbers for more symmetrical peaks. Significant asymmetry in the peaks will result in an underestimation of the variance. Indeed, when theoretical values using this equation were compared to values based on experimental measures of apparent column efficiency, the most asymmetrical peaks deviated the furthest from the theoretical values. Adjusting the cut points in the tails of the asymmetrical peaks significantly improved the correlation between the theoretical and experimental values. From this we can see that care must be taken in determining the boundaries of a peak if an accurate assessment of variation is desired.

Very often the peaks in question deviate significantly from a Gaussian distribution. The Foley-Dorsey equation gives time-based variance:[28]

$$\sigma^2 = \frac{W_{0.1}^2}{1.762(W_R/W_F)^2 - 11.15(W_R/W_F) + 28} \qquad (16)$$

where $W_{0.1}$ is the peak width at 10% height, W_R is the width from the center of the peak to the tail at 10% height, and W_F is the with from the front of the peak to the center. This would accommodate non-Gaussian peaks more accurately for peaks having a (W_R/W_F) ratio of 1.01 – 2.76.[28, 29] In Fountain's study,[15] band spreading measurements were obtained by measuring peak width at 13.4% peak height (4σ) and the degree of tailing was not addressed. Nguyen *et al.*[20]

simplified their calculation of variance by ignoring the contributions from the injector and detector and using the variance from the connecting capillaries as the sole significant source of band broadening. As significant differences in variation due to injection have been observed based on injector mode, injection loop diameter, and injection volume, etc., this does not seem to be an accurate assumption to make. Experimentally, by taking the second central moment of peaks eluted at different temperatures and various flow rates, Nguyen *et al*. found the values to be within 20% of those predicted, which was deemed sufficiently accurate for their purposes.

Experimental conditions used for the measurements will also impact their accuracy. Analysis should be done over a variety of flow velocities, as we have seen that the variance arising from flow in an open capillary is largely dependent on mobile phase velocity. At low velocity peaks tend to be more symmetrical, so when analyzing using an equation that assumes a Gaussian distribution, the results will be more accurate for lower velocities and low-viscosity solvents. At high velocities the extra-column contributions from the capillaries do not continue to increase with increasing velocity as seen previously, however the absolute variance of the eluted peaks is very small, so the extra-column variance can have a larger effect on calculated plate heights.[28] The width of a peak in time decreases as mobile phase velocity increases, so the data acquisition rate should be verified sufficiently high to accurately detect the peaks.[15] Mobile phase velocity effects also depend on the composition of the mobile phase. As mobile phase viscosity is lowered, the molecular diffusion coefficient increases, resulting in faster radial diffusion of the sample across the diameter of the connecting capillary channels.[11] For an accurate evaluation of the band broadening in a system, representative mobile phases should be incorporated whenever possible.

For isocratic elutions, the effect of extra-column band broadening is lower with increasing retention factor (k′) of the analyte because the longer the analyte is retained on the column, the broader the peaks. When studying a variety of analytes, one can either account for the variation in k′ mathematically,[30] or adjust the organic composition of the mobile phase in an attempt to hold k′ constant. In an evaluation of the efficiency of columns, the lower the retention factor, the larger the error in height equivalent to a theoretical plate (HETP) calculations when data are not properly corrected to include extra-column contributions.[6] Moreover, one must consider the effect of temperature on retention factor and adjust either calculations or mobile phase composition accordingly when performing studies where temperature varies.[20] In Guillarme's study[13] a decrease in efficiency at elevated temperatures was often attributed to extra-column band broadening contributions due to low retention factor at high temp and a thermal gradient in the column. In a later study[20] the retention factor was held at ~13 by modifying the proportion of organic solvent and the mobile phase was properly preheated. Under these conditions the minimum plate height remained almost constant when temp increased. This is in agreement with Fountain's assertion[15] that no additional efficiency, in terms of achievable minimum plate height, is obtained when increasing temperature.

It becomes clear, then, that to obtain a true and accurate evaluation of an instrument's extra-column band broadening contributions, care must be used to select the calculation method that most accurately applies to the peak shapes. For best results, a range of flow rates and temperatures should be considered, with careful consideration given to the retention factor of the analyte(s) and the composition of the mobile phase. Ignoring these contributing effects could give significantly misleading estimations of the extra-column variance of a system.

3.2 APPLICATIONS

Calculated extra-column variance values have their most obvious application in evaluation of newer or custom-developed UPLC instrumentation. Existing commercial instruments or custom built instruments can be optimized to minimize band broadening by modifications such as replacing injector stators and components, replacing and minimizing the length of connecting capillaries, optimizing mobile phase and column heating units, and choosing appropriate detector flow cells. Only by minimizing extra-column contributions will the complete benefits of new column technologies be fully realized. Consideration of the instrumental band broadening is critical in obtaining an accurate evaluation of the efficiency of small particle or core shell columns of varying diameters and lengths, and in evaluating the effect of temperature on the analysis.

Kinetic plots have found great application in evaluating the kinetic potential of high-temperature UPLC. Rather than plotting plate height (H) versus linear flow rate as in a van Deemter curve, an unconstrained kinetic plot charts retention impedance times (t_R/N^2_{eff}) versus the effective plate number (N_{eff}). This enables one to compare chromatographic supports of different sizes, shapes, and packings in order to find their best kinetic performance for a given instrument and column.[31] The plots can be constrained by limiting mobile phase velocity, peak width, peak volume, or column length,[17] allowing one to visualize and quantify the impact of extra-column band broadening among other parameters. Plots can be calculated for the column and the instrument, or the instrument variance component can be subtracted to evaluate the column performance alone. These plots show that that while extra-column band broadening affects the performance over the entire range of plate numbers and all temperatures, the effect, as observed by increasing curve height, is strongest in the range corresponding to the narrowest

peaks.[17] In the plots prepared by Fountain,[15] this region corresponded to the shortest columns, which will be most affected by the extra-column effects.

In Heinisch's study[14] it was suggested that an instrument-based kinetic plot is a good way to evaluate instruments to find the best compromise between a small extra-column pressure drop using broad connection tubings and a small extra-column band broadening component obtained with narrow connection tubings. This would be the most effective way to confirm that the assertion made by DeCliq *et al.* that the decrease in available pressure does not significantly negatively impact the efficiency of the separation is also valid for particles smaller than 3µm.

An excellent example of the use of kinetic plots to compare both instruments and column packing materials is illustrated by Fountain *et al.*[15] A plot was made comparing the uncorrected curves for 1.7µm and 2.5µm particles on a UPLC instrument with instrumental variance of 2.8µL and on a conventional but somewhat optimized HPLC instrument with variance of 7.2µL. Low points on the plot correspond to the conditions yielding the fastest critical pair separation.[32] The two relevant equations used to prepare these plots are as follows:[33]

$$t_R = \Delta P_{max} \left[\frac{K_{v0}}{\eta u_0^2}\right]_{exp} (1 + k) \qquad (17)$$

$$N_{eff} = \Delta P_{max} \left[\frac{K_{v0}}{\eta u_0 H}\right]_{exp} \frac{k^2}{(1 + k)^2} \qquad (18)$$

K_{v0} is a velocity based permeability factor. The left side of the plot is dominated by molecular diffusion and the right by mass transfer.

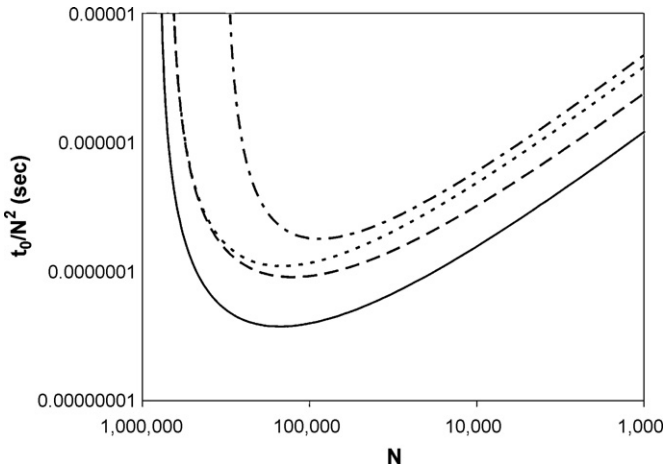

Figure 4. Kinetic plot comparing 1.7µm and 2.5µm particles on instruments with variances of 2.8µL and 7.2µL at 400bar and 1000bar. Solid line – 1.7µm particle, 1000bar, σ_v=2.8µL; Dashed line - 2.5 µm, 400bar, σ_v=2.8µL; Dotted line – 2.5µm particle, 400bar, σ_v=7.2µL; Dashed-dotted line – 1.7µm particles, 400bar, σ_v=7.2µL Reprinted from Journal of Chromatography A, Vol 1216, Kenneth J. Fountain, Uwe D. Neue, Eric S. Grumbach and Diane 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, 5979-5988. Copyright 2009, with permission from Elsevier.

A clear advantage can be seen for the UPLC instrument, and the 1.7µm particles at 1000bar. Interestingly, when comparing the 1.7µm particles to the 2.5µm particles at 400bar, the larger particles have a lower curve on the kinetic plot. This is due to the limitations on the length required to maintain a pressure of 400bar while using the small particle column, and not an indication that the smaller particles have inferior performance.[15]

4.0 CONCLUSION

Significant advances have been made in column packing materials that has, in turn, driven advances in instrumentation. Without a proper understanding of the impact of the extra-column band broadening, the maximum performance of these new columns will not be realized. Both columns and instruments must be evaluated with as much accuracy as possible.

Injectors must be capable of delivering reproducible and small injection volumes with as little broadening of the injected analyte peak as possible. Needle seat capillaries, if used, should have a minimal volume. Stators should be carefully selected to minimize dispersion. The impact of injection method is also a factor in the quality of the injected peak.

Connection tubings are the easiest instrument component to modify. Tubing should have a minimum diameter and length to minimize extra-column band broadening, with the caveat that the pressure drop due to connection capillaries reduces the amount of pressure available to the column. The addition of mobile phase preheating is necessary for high temperature work with all but the narrowest capillary columns. Determining the balance between adequate tubing for mobile phase preheating while minimizing dispersion is a balancing act and may be best served by heating the mobile phase prior to the injector.

Detectors are a non-negligible source of band broadening. Detection in UPLC is complicated by the low volume and fast elution rate of the peaks through the detector cell. Data

collection rates must also be assured to be adequate for the rapidly eluted, narrow peaks, or a deterioration in the accuracy of the data will result.

While conventional HPL analysis seldom required consideration of the extra-column band broadening in an evaluation of column or instrument performance, it becomes critical when evaluating UPLC results. Significant room for error exists in the analysis of the peaks, both in the equations used to calculate the extra-column variance as well as how the peaks are measured. Because peaks eluted without a column are seldom truly Gaussian, approximations must be made that may over or underestimate the degree of variance due to the instrument. The best fitting data is obtained when using conditions most similar to those intended for analysis, as the extra-column contribution relative to the variance from the column changes variables like temperature, retention factor, and mobile phase composition.

When an accurate evaluation of the variance is made, an accurate view of the effects of kinetic parameters can be obtained. These plots are valuable tools for the evaluation of column packing materials, diameters, pressures, and temperatures that had previously been outside the practical range of chromatographic instrumentation. Assuming the developments in column materials continues apace, the extra-column considerations will continue to be a factor not to be ignored.

APPENDIX A

DEFINITION OF VARIABLES USED

A	van Deemter eddy dispersion
B	van Deemter axial diffusion
C	van Deemter resistance to mass transfer
D	Diffusion coefficient
d_p	Particle diameter
F	Flow rate
H	Height equivalent to a theoretical plate
k'	Retention factor
K_{v0}	Velocity-based column permeability
L	Length
N	Number of theoretical plates
P	Pressure
Pe	Peclet number
r	Radius
$t_{1/2}^{f}$	Front elution time at half height
$t_{1/2}^{r}$	Rear elution time at half height
t_R	Retention time
U	Mobile phase velocity
u	Average mobile phase velocity
V	Volume
W	Peak width
η	Mobile phase viscosity
σ^2	Variance
φ	Flow resistance

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