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## Gas Chromatography Ovens

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*The transit of peaks through a gas chromatography (GC) column depends strongly on the thermal profile they encounter on the way. Gas chromatographers primarily rely on the classic hot?air?bath type of oven, but several alternatives are also in use. This instalment examines ovens for GC in several forms plus how oven thermals affect peak retention behaviour.*

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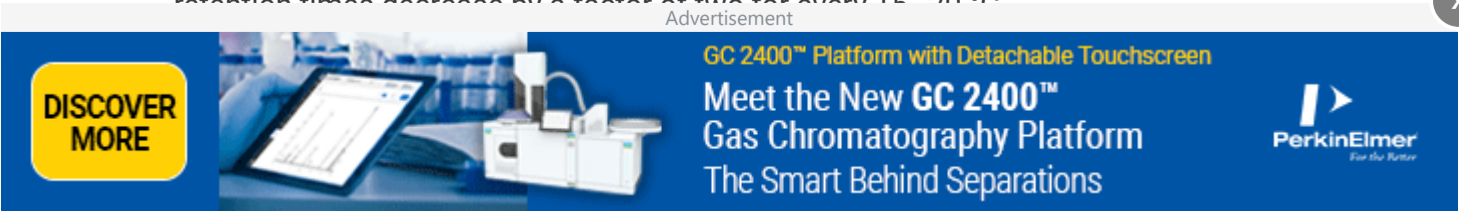
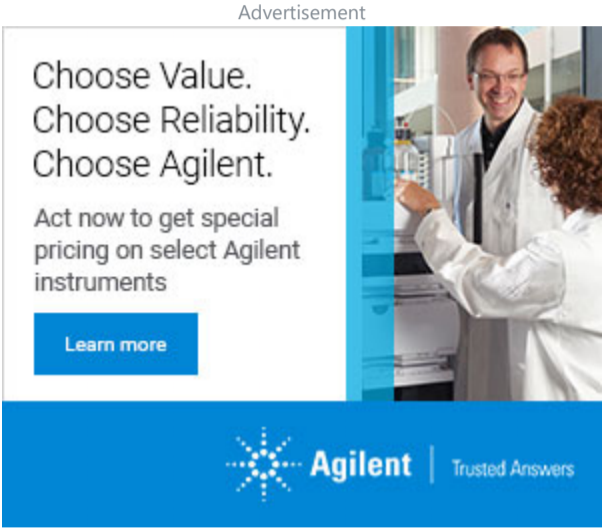
**The transit of peaks through a gas chromatography (GC) column depends strongly on the thermal profile they encounter on the way. Gas chromatographers primarily rely on the classic hot-air-bath type of oven, but several alternatives are also in use. This instalment examines ovens for GC in several forms plus how oven thermals affect peak retention behaviour.**

Gas chromatography (GC) ovens were not developed for cooking purposes, but rather to provide a suitable thermal environment for the elution of chromatographic peaks from GC columns. That being said, I have seen a number of inappropriate applications for GC ovens including reheating of cold pizza for lunch and the warming of casseroles for company potluck dinners. Some of us have even been known to trick out the oven door switch and use an idle GC system as a room heater on cold days.

GC ovens have gone through an evolutionary development just like other GC components have. Today, the majority of GC ovens use rapidly circulating air inside an insulated enclosure to transfer heat to and from the column, much like the convection ovens found in many high-end kitchen ranges. Some readers may be familiar with other means that chromatographers use to control the thermal environment surrounding a column; some of these are quite ingenious. First, though, let's take a look at what kind of thermal conditions a column needs to deliver excellent separation performance.

### GC Oven Requirements

In gas chromatography, the relationship between peak retention and column temperature is fundamental. Changes in temperature cause significant exponential retention shifts, so GC instruments control solute retention times by carefully managing the column temperature along with the column pressure drop. In general, retention times decrease by a factor of two for every 15–20 °C





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times by a small amount from one run to the next. Slightly higher overall oven temperatures cause peaks to be eluted a little earlier, and lower temperatures shift retentions to longer times. In addition to changes in the average oven temperature from one run to the next, other important temperature variabilities include spatial fluctuations along the column because of temperature gradients in the GC oven and dynamic short-term oven temperature fluctuations because of electronic and software temperature-control processes. If large enough, such spatial and short-term changes will affect peak shapes and resolution.

### Run-to-Run Average Temperature Deviations

In general, run-to-run retention time deviations should stay within a window of about  $\pm 10\%$  of the peak width at base. That way there is little ambiguity concerning peak identification by absolute retention time, including for partially separated peaks. The narrowest practical peak widths will put the most demands on the oven temperature repeatability. Relatively short, 10-m long, 0.20-mm i.d., thin-film capillary columns elute significantly retained peaks - those with retention factors of two or greater - with base widths of about 1.5 s minimum. This figure assumes that the column operates at an optimum average linear helium carrier gas velocity of about 30 cm/s with the best theoretical efficiency. Chromatographers can obtain narrower peaks by operating columns at higher than optimum carrier-gas velocities, or by choosing narrower or shorter columns - in other words, by going to "fast" GC. But in general this is about as fast as peaks go for the vast majority of GC systems in operation today.

The oven is not the only factor that contributes to run-to-run retention time variations. The pneumatic system also influences retention times significantly, and the data handling system introduces other uncertainties including its determination of the exact run start time and the peak apex or centroid. I assembled a statistical analysis of these factors and the GC oven using typical values for modern GC instrumentation and columns. Interested readers can refer to a website for this supplemental information (1). In this analysis, the room temperature and GC line voltage supply are assumed to be stable and to not influence the actual oven temperature and column pressure drop. This is not always the case, of course, and such problems fall into the category of instrument "environmental" difficulties that should be resolved before attempting to obtain the best retention time reproducibilities.

**Isothermal Elution:** According to this analysis, the average oven temperature needs to repeat from run to run within less than  $\pm 0.05^\circ\text{C}$  to hold retention times inside a window of  $\pm 10\%$  of peak base widths, for commonly encountered solutes. Lee, Yang, and Bartle cite a similar requirement in their 1984 book (2). These figures are for typical test mixture solutes separated at moderate isothermal temperatures on short narrow-bore thin-film capillary columns, which represent the more difficult cases. Columns with thicker stationary films, columns that are longer, wide-bore columns, and, of course, packed columns generate broader peaks and may tolerate a less stringent temperature environment while still delivering retention time repeatability that is adequate for peak identification purposes. On the other hand, fast GC separations performed on microbore

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but with single-ramp programmed temperature elution the peak widths stay approximately constant across a GC run, for peaks that are eluted during the temperature ramp. Most peaks are essentially immobilized upon injection at the initial column temperature; as the oven temperature increases, solutes begin to move along the column. Each spends about the same time moving through the column (3), which results in the peaks having approximately the same widths as they are eluted. For temperature programming, oven temperature ramps need to repeat the time and temperature curve with the same precision as required for isothermal elution. The oven heating system must have sufficient power to maintain a linear programme ramp from the start temperature to the final temperature. The maximum rate at which the oven temperature can increase linearly is related to the thermal mass of the oven cavity, including the column, the power that the heater can dissipate into the oven, the efficiency of the oven boundary insulation, and the differential temperature between the inside of the oven and the external environment.

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One principal difference between isothermal and programmed elution is the slight time lag between the actual column temperature and the setpoint temperature of the temperature ramp. As the oven temperature controller increases the setpoint temperature according to the demands of the programme ramp, the actual oven temperature lags behind by a small amount and the temperature inside the column lags a little more. The temperature lag is larger at higher programme rates. This temperature differential is a necessary by-product of both the nature of temperature-control algorithms and the physics of heat transfer from the heater to the column. For our purposes, it is the temperature in the column itself that counts.

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The electronics and software must repeat not only the same time and temperature profile across the run, but also the thermal conditions in the oven must be consistent. This is one reason why temperature-programmed ovens require a temperature equilibration period after cooling down before a new run can commence. Shortly after the oven cools down, the oven temperature probe may indicate the initial oven temperature, but there is still a significant amount of heat remaining in the oven; the walls and the column can be significantly hotter than the indicated temperature. This is the reverse of the temperature lag encountered with oven heating, and good practice dictates that sufficient time be allowed to dissipate the residual oven heat and bring the column as close as possible to the initial oven temperature. An equilibration time of 2–4 min is typical for most air-bath GC ovens with temperature programming capability. With isothermal elution, no equilibration time is required after the oven reaches its temperature for the first time, because the temperature is not changed during the run.

**Oven Gradients:** A high degree of run-to-run average temperature repeatability ensures the best retention time precision. GC ovens also must maintain a uniform and stable temperature profile across the column during elution. The process of solute partitioning and migration along the column averages out temperature gradients so that peaks are eluted as if they experienced the average column temperature along the length of the column. Minor temperature

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affect the average temperature that solutes experience.

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Some temperature gradients are normal and necessary. The column inlet and outlet connect to the injector and detector, which are normally at elevated temperatures relative to the oven. Where GC column ends are positioned inside the inlet and detector, a negative temperature gradient exists from the inlet into the column and a positive gradient out into the detector. The net effect on retention is negligible, since solutes will be weakly retained, if at all, in the short sections of heated column ends, which act more like connecting tubing. Gradients also exist close to the oven walls because of heat losses or gains from cooler oven walls or warmer heated inlets and detectors. In a conventional air-bath oven, the column is normally positioned in the centre of the oven to immerse it in the most thermally uniform area. These stable temperature gradients do not seriously affect performance if they are stable both during a run and in the long term from run to run.

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Gradients that fluctuate from run to run will affect retention time stability, but gradients that fluctuate rapidly during a run will affect peak shapes. A poorly sealed air-bath oven can generate this type of gradient. A controlled amount of air normally circulates through an air-bath oven, to prevent the buildup of explosive hydrogen gas. This is done in such a way that significant temperature gradients are not generated. But if the oven door or vent isn't sealed properly, significant amounts of cold air can enter and blow directly on the column and create a rapidly fluctuating localized temperature gradient. For fused-silica columns in particular, with their thin walls and rapid thermal time constants, fluctuating gradients will alternately trap and release portions of solute bands as they pass by, which will distort the peak shapes and may even split peaks up into apparent multiples.

GC operators play an important role in ensuring the minimum possible gradients, by installing columns correctly. As much as possible, columns should be centred within the column oven, with no length of the column closer than about 1 in. (2.5 cm) from the oven walls, except of course for the inlet and detector connections. Column mounting brackets provided by the manufacturer are the best way to provide secure and centred column mounting.

## Oven Heating Schemes

Over the years, GC researchers have envisioned and built a wide variety of column heating schemes. Early workers were well aware of the requirements imposed by the sensitivity of GC retention times to temperature changes and by the problem of transient thermal gradients. They had it easier than present-day chromatographers, however, because they had to accommodate packed columns and metal or thick-walled glass capillary tubing, but not fused-silica tubing. Thin-walled fused-silica tubing responds more rapidly to oven temperature changes and is more susceptible to the effects of rapidly changing oven gradients. The newest generation of GC ovens includes features designed to maximize performance with all columns.

Oven heating schemes fall into two broad categories: those that rely on a fluid to transfer heat to and from the column, and those that use

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instruments because of their low power requirements and good long-term stability. Columns installed inside resistively heated metal tubing or directly heated column tubing, suspended inside a conventional air-bath oven, deliver very fast programming rates for high-speed GC.

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**Fluid Ovens:** The earliest report I could find of a fluid oven for GC involves the suspension of the column inside a vapour bath over a boiling pure substance (4). This technique of temperature regulation had been used in various related disciplines even before partition GC came into its prime in the 1950s, and it was a natural step to use it for the then-new technique. The operator could change the temperature of the vapour bath up or down by regulating the pressure inside a sealed oven container. For example, *p*-xylene boils at 138 °C at 1 atmosphere and temperatures down to about 30 °C lower were attainable at reduced pressures (5). Changing the oven temperature outside this limited range was not very convenient because the pure substance had to be exchanged for another with a higher or lower boiling point. This arrangement produced a fairly stable thermal environment for the column, as long as the atmospheric pressure didn't change too much. There were also problems with decomposition of the boiling substance at higher temperatures, as well as the flammability of the vapours, and chromatographers abandoned this heating system fairly early.

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Oil baths were also used as oven thermostats (6). After the operating temperature was reached, if the heating vessel was well insulated and efficiently stirred, a very uniform thermal environment was possible. Temperature control circuitry provided fairly good temperature stability. Such baths could be temperature programmed, but at a limited rate because of the large heat capacity of the oil. A main drawback of oil bath ovens was the inconvenience of working with high-molecular-weight oils that would coat the column and connections. The slightest leak would permit the oil to enter and destroy the column or, at the very least, drastically modify its retention characteristics. Changing the column was difficult and messy. A water bath was also used, but with a limited temperature range.

**Air-Bath Ovens:** GC researchers understood at the onset that an air-bath oven was potentially superior to liquid or vapour bath designs. The temperature in an air bath could be varied rapidly over a wide range without changing the working fluid, and it would be much easier to change columns. However, early air-bath oven designs from the 1950s suffered from large temperature gradients and a significant lag time between the oven setpoint and actual column temperatures. Improved fans and better insulation helped to remedy the situation, and by the mid-1960s the air-bath oven was almost universally accepted. The subsequent introduction of microprocessor temperature control in the 1970s and the adoption of a number of other important features - including improvements made specifically for fused-silica columns - resulted in modern GC oven designs that are ubiquitous today. GC air-bath ovens are available in both cylindrical and cubic forms.

Common features among air-bath ovens include high-speed fans for turbulent mixing that minimizes internal gradients. The columns and temperature sensor are shielded from direct thermal radiation

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capable of linear temperature programming rates up to at least 75 °C/min with a standard heating element. However, the maximum linear programme rate declines to around 20–30 °C/min at temperatures above about 175 °C because of increasing heat losses through the temperature gradient, through the oven insulation, and out to the surrounding environment. More powerful oven heaters available with some instruments can yield higher programming rates up to 120 °C/min that are suitable for higher speed GC separations. When the columns occupy only a fraction of the full oven volume, even faster heating and cooling are obtainable by reducing the oven air volume with an internal insulating blanket.

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The fastest GC total run-to-run times require not only fast heating, but also rapid cooling back to the initial oven temperature. The use of an extra fan that forces cooling air through the oven vents helps to speed cool-down time. Cryogenic cooling is another option that minimizes cool down, but is not always convenient or available.

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**Solid State Ovens:** GC experimenters also developed a number of different solid-state column heating schemes over the years. An early design used heating wire that was simply wrapped around a straight or U-shaped packed column and insulated with asbestos tape. A variable voltage gave rudimentary temperature control (7). Another approach connected a source of electrical current across the metal packed column itself, and at least one commercial GC used this method (8). This approach has also been applied to metallic open-tubular columns and metal-coated fused-silica columns (9).

A different solid-state column heating design packages the column and a separate heater into a monolithic block. One or more metal capillary columns are wound with a heating element around a core and the entire assembly is then encased in thermoplastic resin. The column tubing terminates outside the oven assembly in adjacent valves, inlet systems, and detectors, and the entire assemblage is placed inside an insulated enclosure. This type of construction is attractive for portable isothermal GC systems because once heated, it doesn't require a lot of power to maintain temperature and it can be very small in size. Changing the column requires a complete exchange of the inner oven assembly, however.

**Micro GC:** GC systems that use nonconventional micromachined inlet, column, and detector modules have experienced significant development in the past decade. Such devices may use columns etched into a planar substrate with integral or attached heating elements. Small both in size and power consumption, these GC systems are finding a niche in field portable applications.

**Hybrid Ovens:** Some of the most recent high-speed GC arrangements for benchtop instruments use a hybrid combination of an air-bath that surrounds solid-state heated columns. By using short columns with smaller internal diameters in combination with fast heating, such a device can deliver the resolution of longer, larger diameter columns in less time. In one arrangement, the column runs coaxially inside a coiled, externally insulated metallic tubular heater that is suspended on a cage in the air-bath GC oven. Such devices can achieve ramp rates up to 1200 °C/min, although at such rates the oven temperature will likely get ahead of or escape the

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methodologies for controlling column temperature. Far removed from simple cake baking requirements, the physics and chemistry of GC separation and elution determine the requirements for temperature control. The need for application flexibility or specificity determines the kind of heating system that is appropriate. A broadly applicable system such as the conventional air-bath oven gives the greatest flexibility at the expense of portability, power consumption, and space. Dedicated systems in which the column and heater are permanently mated yield low power consumption, portability, and compactness. Interest in high-speed and high-resolution GC will continue to spur developments in column heating technologies.

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## Modern Supercritical Fluid Chromatography — Possibilities and Pitfalls

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*There has been a revival of supercritical fluid chromatography (SFC)*

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**There has been a revival of supercritical fluid chromatography (SFC) in recent years, especially in the chiral preparative field, but also more recently in the analytical area. However, SFC is considerably more complex than liquid chromatography (LC), mainly because of the compressibility of the mobile phase. One can say that SFC is a "rubber variant" of LC where everything considered constant in LC varies in SFC. In this review, we go through advances in theory, instrumentation, and novel applications.**

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Recently, both instrumental manufacturers and the pharmaceutical industry have increased their interest in supercritical fluid chromatography (SFC) because of the lower environmental impact and the considerably shorter separation times compared to traditional high performance liquid chromatography (HPLC). The use of low-viscosity carbon dioxide as the main solvent enables operation at higher flow rates as compared to liquid chromatography (LC) (1), and the low toxicity of this mobile phase and its ease of recycling have created another important incentive. Even though the SFC revival has focused on preparative chiral separations (2), it has also been successfully used in many achiral applications. However, LC still dominates analytical applications because of its versatility and, so far, its superior robustness compared to SFC. If the reproducibility of modern SFC could be considerably improved, we believe its revival would be strongly boosted.

The inherent problem with SFC that makes it so much more complex than LC is the compressibility of the mobile phase, which makes SFC a "rubber variant of LC". Everything considered as constant in LC is not constant in SFC (3,4). This ultimately results in radial and axial density and temperature gradients in the column that affect the thermodynamics of adsorption and cause a volumetric flow rate gradient through the column (5–7). The practical consequences are serious. We cannot be guaranteed that the set operational conditions reflect the true conditions over the columns like we can in LC. This results in poor system-to-system reproducibility and transfer between analytical methods, which in turn hampers the wider use of SFC in the pharmaceutical industry. These phenomena also result in poor predictions in scaling up from analytical SFC instruments to preparative SFC instruments (8). The consequence is that optimization in process- and preparative-scale SFC must be done in the large scale, which costs money and energy and may even be impossible in some cases. Therefore, a proper understanding of how operational parameters such as pressure, temperature, and solvent composition affect the separation and its reproducibility is of paramount importance.

In this brief investigation, with both new and review material, we start out with some illustrative examples of poor method transfer among different analytical systems. Thereafter, we report on recent investigations on which experimental parameters are the most important to improve reproducibility and method of transfer in SFC. Some guidelines to users and some advice for improvements to manufacturers is also provided.

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reproducibility between different SFC systems was studied by injecting 20  $\mu\text{L}$  (full-loop) of 350 g/L antipyrine solution on four different SFC systems using identical instrumental settings and the same column (see Figure 1). Liquid carbon dioxide was supplied to systems 1, 3, and 4 (AGA Gas AB, Sweden) and system 2 was fed gaseous carbon dioxide. The same 150 mm  $\times$  4.6 mm Kromasil silica column, packed with 5- $\mu\text{m}$  nominal particle size, 100- $\text{\AA}$  pore size media (AkzoNobel Eka) was used for all systems. The back pressure ( $P$ ) was 150 bar, the column temperature was 35  $^{\circ}\text{C}$ , and the eluent composition was 85:15 (v/v) carbon dioxide–methanol. All experiments were carried out at volumetric flow of 2 mL/min.

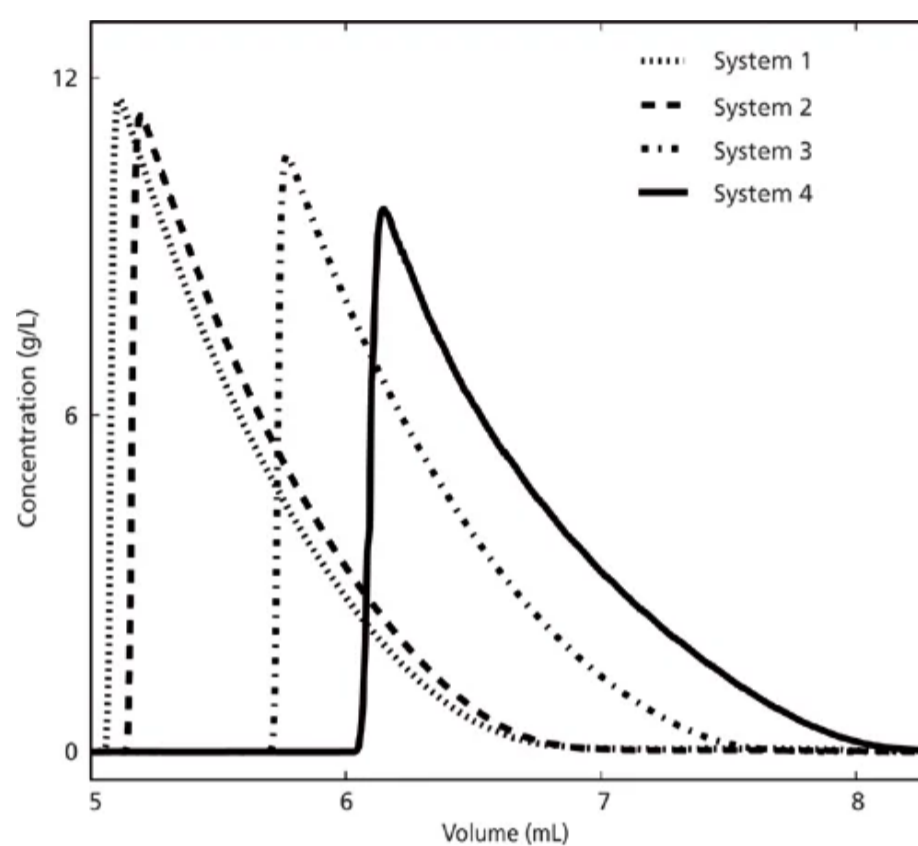


Figure 1: Experimental chromatograms recorded on the various SFC systems. The set volumetric flow was 2 mL/min and the injection volume was 20  $\mu\text{L}$  of a 350-g/L sample.

The resulting overloaded elution profiles, corrected for system void volumes, is clearly system dependent as can be seen in Figure 1. This figure also shows that the elution volume is larger on system 4 while systems 1 and 2 have lower, and similar, retention volumes. However, system 3 (same instrument model as system 1) had a retention volume that was more than 0.5 mL larger than that of system 1 when using the same column and the same set conditions. This observation indicates that either the set conditions are not manifested in the systems or that the operational column conditions were different, although identical set values were used. We further investigated if the volumetric flow rate was the reason for the observed difference between the elution profiles shown in Figure 1, but the column dead volume was determined and was estimated to be approximately 1.9 mL on each system. This observation means that the volumetric flow rate was more or less identical on all systems; otherwise the void volumes would have been different.

Other important parameters governing retention are the density of the mobile phase, the fraction of organic modifier ( $C_M$ ), and the column temperature ( $T$ ). The density is a nonlinear function of pressure ( $P$ ) and temperature; to calculate the density of the carbon dioxide and methanol stream inside the column, the pressure at the

will be divided between capillaries and column. Pressure drop from the capillaries will depend on their length and diameter, which cannot be assumed to be identical between the different systems. So the reason for the problem is probably due to the fact that in SFC - in contrast to LC - the set operational values are not necessarily identical with the actual operational values.

### Deeper Investigation of the Causes of the Poor Reproducibility

**Between Systems:***Initial Experiments:* We used a design-of-experiment (DoE)-based approach (9) to find out the most important parameters affecting the reproducibility of retention for different neutral model compounds in this system and similar ones. In DoE, the effects of variations of operational parameters on a certain response, such as retention factors or selectivity in chromatography, are statistically investigated under controlled experiments. For this particular study, we had to find out the real temperature, pressures, and the cosolvent fractions inside the column. Thus, we used external devices to measure the real mass flows over the column and external sensors for measuring temperature and pressure at different positions along the column (5).

Before the DoE-based experiments and modelling, we investigated experimentally how retention volumes are affected by changes in system back pressure and modifier content (10). Analytical injections of 5  $\mu$ L of 0.350-g/L antipyrine solutions were performed at back pressures of 140–160 bar and modifier fractions of 14–16%, on system 1 (see Figure 2). As seen in Figure 2, the retention volume was strongly affected by changes in the modifier concentration and to a smaller extent to the "set" back pressure. When performing the system reproducibility experiments described in the previous section, we noticed that system 4 had a much lower pressure drop compared to systems 1 and 2. This sensitivity and back pressure difference between systems probably explains the observed differences in retention volumes mentioned earlier.

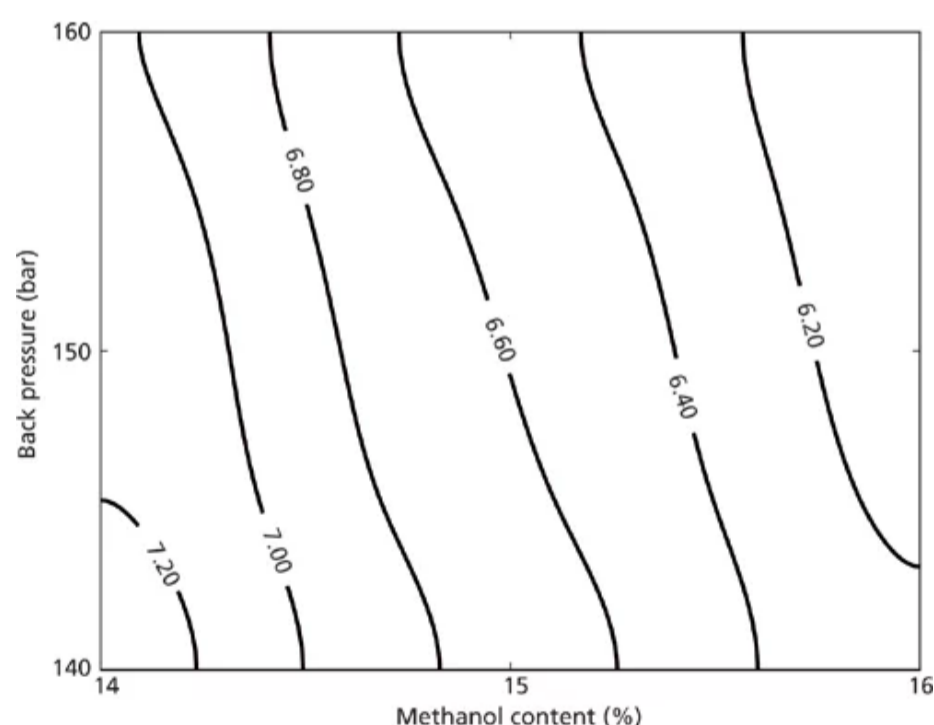


Figure 2: Retention volume of analytical peaks as a function of methanol content and pressure determined using 14, 14.5, 15, 15.5, and 16 (v/v) % methanol content at 140, 145, 150, 155, and 160 bar on system 1. All analytical injections were 5  $\mu$ L of a 0.35-g/L sample.

*Design of Experimental-Based Investigation:* We used DoE to investigate the impact on retention by cosolvent pressure, and

removing statistical outliers and then removing any statistically insignificant terms from the polynomial at a 95% confidence level.

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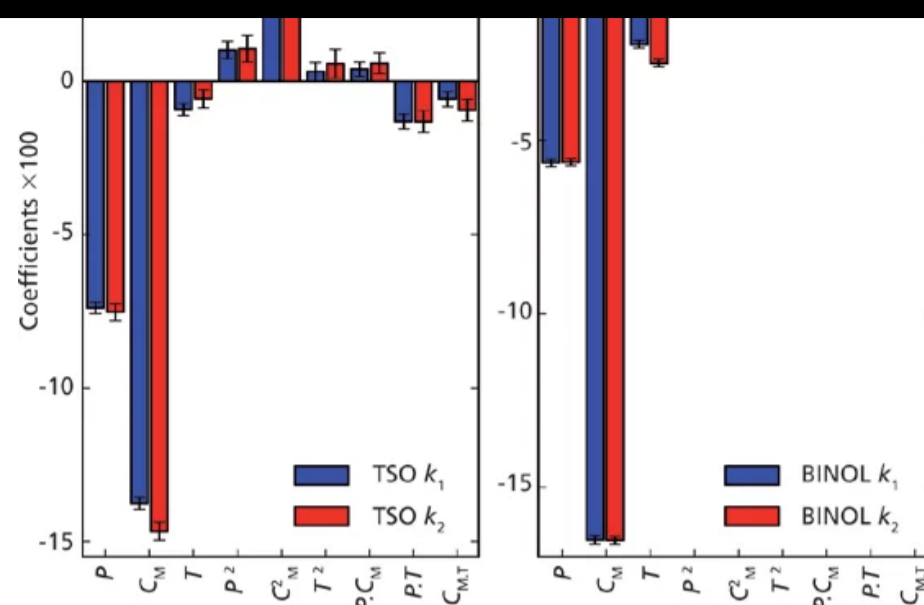
To study the dependency and variation of common chromatographic parameters such as retention factors, selectivity, and productivity with parameters such as amount of cosolvent, temperature, and pressure, the true parameter values in the chromatographic column must be used rather than the values set on the instrument. The true values were determined by using external measurements of pressure, temperature, and mass flow. To verify the set volumetric flow rate, the corresponding density of the mobile phase fluid was calculated using the Kunz and Wagner equation of state as implemented by the National Institute of Standards and Technologies in a database software used in science research (11). Interestingly, the deviation between the set and estimated volumetric flow rate, temperature, and pressure was found to be relatively small for most experimental conditions used.

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*Parameters of Importance for Retention Factors:* Before a successful method development can be performed in SFC, it is essential to have knowledge about and to be able to predict how the retention factor is affected by a certain change of the operating parameters (that is, the variables). The centred and normalized coefficients for the retention factor models are presented in Figure 3. Here the test solutes studied were *trans*-stilbene oxide (TSO) and 1,1'-bi-2-naphthol (BINOL). In particular, Figure 3 shows the impact of different operation parameters on the retention factors for the first and second enantiomer of TSO and BINOL. The height of positive or negative bar in the plot shows the relative degree of impact of the corresponding operational parameter. The operational parameters are denoted in the x-axis: pressure ( $P$ ), methanol fraction ( $C_M$ ), and column temperature ( $T$ ); significant values of the quadratic and more complex term on the x axis indicate nonlinear dependencies. In Figure 3, it is clear that the volumetric  $C_M$  was the most important parameter, which is represented by the largest coefficient value for methanol. The methanol fraction also had a large quadratic term ( $C_M^2$ ), meaning that the methanol dependence is not linear. Pressure was the second most important factor for the retention factor, and the retention factors all have similar pressure dependencies with a slight nonlinearity (see Figure 3). In Figure 3, it can also be seen that the interaction terms are all significant, that is, the coefficients of the interaction terms are shown to be significant. These interaction terms would be missed if one factor at a time were varied. Interestingly, the effect of temperature in the investigated region was low for both components enantiomers, however, slightly larger for BINOL (see Figure 3).

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Figure 3: Centred and normalized coefficients from the model fit for the first and second retention factor, respectively, for (a) TSO and (b) BINOL. The error bars represent the 95% confidence interval of the coefficients. The height of a negative or positive bar in the plot shows the relative degree of impact of corresponding parameter. Adapted with permission from reference 9.

Figure 4 contains contour plots illustrating the combined dependency of the retention factors on methanol fraction and pressure for TSO and BINOL, respectively, at the fixed column temperature 30 °C. For both enantiomers of both components, we can see that the larger the methanol fraction and the larger the pressure the lower (more blue colour) the retention factors (see Figure 4). The highest values of the retention factors are obtained for the second enantiomer (for both TSO and BINOL) at combined low methanol fractions and low pressures (deeply red colour). It is evident that the retention factors for both solutes have similar trends when studied in the methanol–pressure plane, despite the different density variations seen between the experimental conditions for TSO and BINOL.

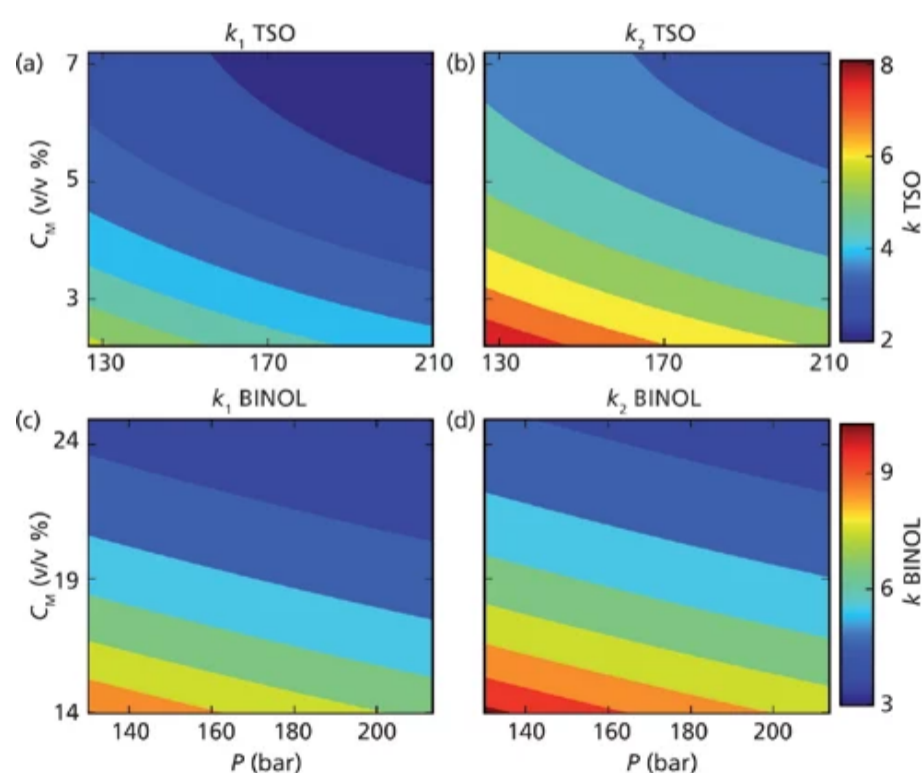


Figure 4: Contour plots showing the retention factor ( $k$ ) as a function of pressure and amount of methanol in the eluent: (a)  $k_1$  for TSO, (b)  $k_2$  for TSO, (c)  $k_1$  BINOL, and (d)  $k_2$  BINOL. Adapted with permission from reference 9.

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This is achieved by screening combinations of chiral stationary phases (CSPs) and cosolvents. Typically, selectivity is maximized while  $k'$  is minimized. Loading studies utilizing maximum sample concentration on suitable candidate systems can then be performed. In this study, the dependency and variation of productivity on cosolvent, pressure, and temperature was studied. Either of the enantiomers of TSO was selected as target. The optimal chromatogram was chosen to maximize injection volume while maintaining touching-band separation (that is, having a yield of 100%). The productivity was calculated by assuming that stacked injections could be performed for the separation of either enantiomer of TSO at 30 °C, 160 bar, and 5% methanol. The trends in productivity were very clear and analysis of parameter importance revealed that the most important factor to increase productivity was to increase the fraction of cosolvent followed by an increase of temperature (see Figure 5[a]). Therefore, by simultaneously increasing methanol content and temperature, the productivity in this case could be efficiently maximized as can be seen in Figure 5(b). By decreasing pressure, the productivity could be further increased but only marginally (data not shown). It should be noted that a complete optimization would also entail further work on the volumetric flow or concentration of the sample, but that was beyond the scope of this study. Interestingly, the selectivity for the separation of TSO increases with decreasing methanol content. Hence, in this case, maximizing selectivity will yield lower productivity.

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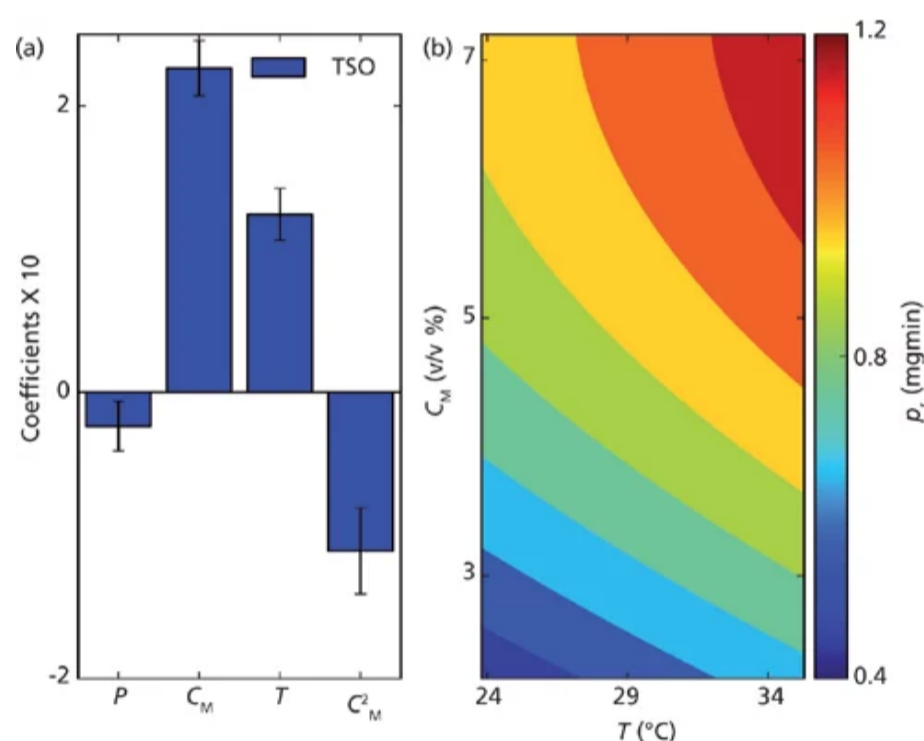


Figure 5: Centred and normalized coefficients with 95% confidence interval from the model fit to the productivity for the optimum touching-band chiral separation of TSO are plotted. In (b) the productivity is plotted as a function of the amount of modifier in the eluent and the temperature. Adapted with permission from reference 9.

## Conclusions

This study shows that SFC is a complicated chromatographic method and that the same instrumental set conditions do not necessarily give the same actual working conditions over the column on different systems for a variety of reasons. Recorded elution profiles for the model compound antipyrine injected onto the same silica column on three different systems at the same set conditions

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and then the selected method is transferred to a larger preparative system. A design of experimental approach was used to study the impact of a change of the most common operational parameters using neutral model compounds on mostly chiral systems. The model systems presented here - except for antipyrine separated with a silica column - consisted of racemic mixtures of TSO and BINOL and were separated using a chiral cellulose-derivative silica-based column. All input parameters were carefully measured using external sensors. The parameters investigated were column temperature, pressure, and cosolvent fraction. The methanol fraction was the most important factor for governing the retention factors, and the pressure was the second most important factor. On the other hand, the temperature did not have a significant effect on the retention factor. Increasing either the temperature or the pressure resulted in reductions of the retention factors. Although not reported here, this result was also obtained with another chiral system studied (racemic 1-phenyl-1-propanol as an analytical model compound) (10).

For preparative purification of either enantiomer of TSO, the most important parameters were found to be methanol content and temperature. The selectivity for the separation of TSO increases with decreasing methanol content. Therefore, in this case, maximization of the selectivity will yield lower productivity. As in most forms of chromatography, compromises must also be made in SFC.

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# Listen to the Data

Published on: March 1, 2015

John W. Dolan

LCGC Asia Pacific

LCGC Asia Pacific,



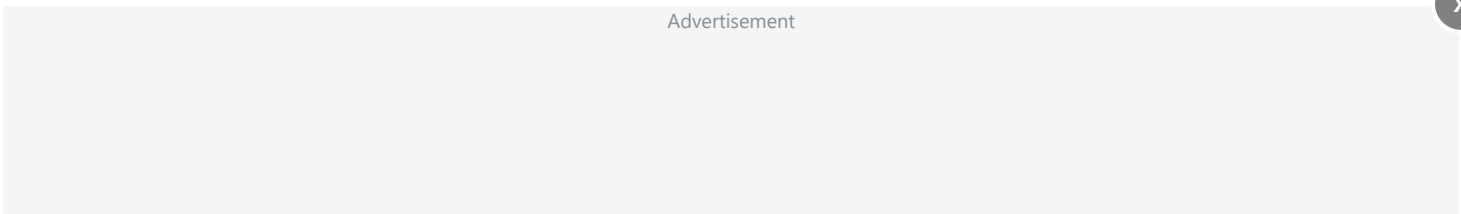
A stepwise process is described to help isolate and identify the cause of a method failure.

Volume 18 Number 1  
Pages 17-21

## A stepwise process helps isolate and identify the cause of a method failure.

One of the most frequent times that we discover a problem with a liquid chromatography (LC) method is when we examine a data set following the analysis of a batch of samples. This month's "LC Troubleshooting" looks at some data submitted by a reader who suspected that something wasn't right with their results. These data give us a good example of how we can examine data for abnormalities and formulate some experiments to try to identify the problem source so we can correct the problem. I have somewhat obfuscated the details so that the reader and company remain anonymous. The sample comprises a pharmaceutical formulation that was being assayed for potency following a particular stress test. A single batch of product was divided into 12 samples, which were then treated in the same manner. For analysis, two subsamples were weighed from each sample, diluted, and injected, for a total of 24 sample injections. The potency was determined by comparing the area response of each injection to the response of a reference standard. The method stipulates that if the two subsamples disagree by more than 1.0% in assay value, the source of disagreement must be investigated. The reader reported that normally these "duplicate" samples agree within 0.5%.

The data I received are listed in the first two columns of Table 1. Each sample is numbered, and the associated letter identifies the subsample (for example, 1a and 1b are subsamples of sample 1). I have noted the absolute difference between the two subsamples in the third column. The abnormality that triggered the reader's inquiry was the 1.41% difference between samples 4a and 4b. This difference exceeded the limit allowed by the method and required



Sample*	% Assay	Difference†
1a	86.18	0.49
1b	85.69	
2a	86.45	0.45
2b	86.90	
3a	86.10	0.55
3b	86.65	
4a	86.11	1.41
4b	87.52	
5a	85.81	0.14
5b	85.67	
6a	86.64	0.73
6b	85.91	
7a	86.18	0.14
7b	86.32	
8a	86.68	0.24
8b	86.44	
9a	86.58	0.07
9b	86.51	
10a	86.83	0.59
10b	86.24	
11a	86.58	0.45
11b	86.13	
12a	86.16	0.19
12b	85.97	
Average	86.34	
SD	0.42	
%RSD	0.5%	
*Samples 1–12 are divided into subsamples a and b, which should be equivalent. †Difference in assay value between subsamples a and b of each sample.		

Table 1: Percent assay values for individual injections of a pharmaceutical product.

### Initial Examination

When I try to solve a problem like this, I like to examine the data in several ways. Often I find that a table of data, such as that of Table 1, makes my eyes glaze over. I do much better with a graphic representation. To get an idea of how atypical the 1.41% difference is, I constructed the frequency plot shown in Figure 1. Here I simplified the data set by "binning" the absolute differences into groups with 0.25% increments, so you can see, for example, that there were five samples in which the difference between injections was 0–0.25%. All the data points except the 1.41% value were <0.75% difference.

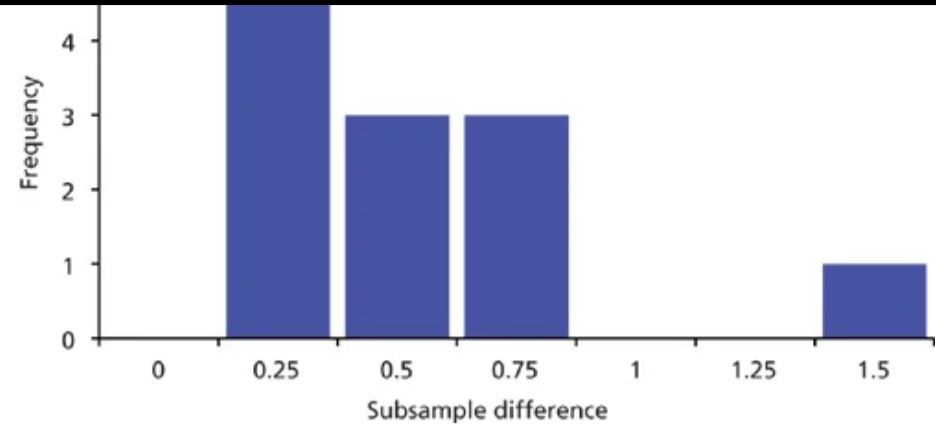


Figure 1: Frequency distribution of subsample differences from Table 1, binned into 0.25-unit increments.

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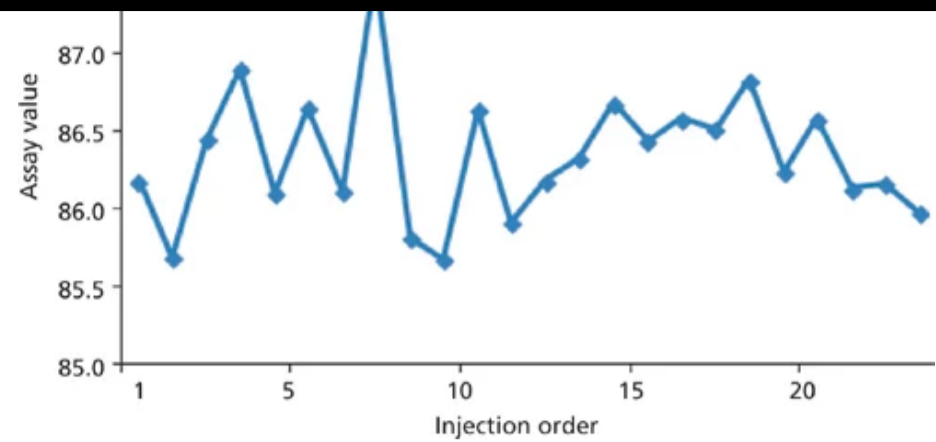
The big gap between the 11 good sample pairs and the one bad one makes the problem pair seem like an obvious outlier. But is there any more quantitative measure of this? One simple technique to test for outliers is the Dixon's Q-test. A test value is calculated as:

$$\frac{|\text{suspect} - \text{nearest}|}{(\text{largest} - \text{smallest})} \quad [1]$$

For this example,  $|1.41 - 0.73| / (1.41 - 0.07) = 0.51$ . The critical value of Q for  $n \geq 10$  is 0.464 (1), meaning that any test value larger than the critical value is an outlier. Now we have some statistical support in stating that the difference in assay values for sample 4 is an outlier. As I look at the results of the Q-test, however, it looks to me like the 1.41 value isn't very much of an outlier. I checked this by looking at different suspect values using the data set of Table 1, and it is easy to show that the critical value is exceeded only when the suspect value is  $>1.3\%$ . This says to me that if the current data set is typical for this method, the requirement for differences of  $<1.0\%$  may be a bit too tight. That is, a value  $>1.0\%$  will trigger an investigation, but unless it is  $>1.3\%$ , it is not likely that it can be proven an outlier with the Dixon's Q-test. Such limits should be set as part of the method validation, where large data sets are available and the normal variation of the method can be determined more easily than with the limited data available here.

### Digging a Bit Deeper

Sometimes it is useful to examine the data for any trends that might be obvious. An easy way to make a first pass at this is to simply plot the assay values over time. In Figure 2, I have plotted the assay values in order for the 24 injections. There doesn't seem to be any trend to larger or smaller values over the course of the analysis. The variability for the first 12 injections seems to be larger than for the last 12, but these were run on two separate days, so it may be a day-to-day difference as much as anything. The overall variability in the data is shown at the bottom of Table 1 with the percent relative standard deviation (%RSD) of only 0.5%. Considering that many autosamplers have %RSD in the 0.3–0.5% range using reference standards under carefully controlled conditions, it looks to me like this method (including the autosampler) is operating with acceptable precision.



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Figure 2: Plot of assay values from Table 1 in injection order.

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Although the %RSD is good when comparing samples, I wondered how good the precision was for the same sample with multiple injections. I asked the reader if such data were available, and I was supplied with the data in Table 2. In Table 2, I have shown the results for the original data from Table 1 (4a and 4b), the reinjection data of the same vials (4a-ri and 4b-ri), and the transfer data of the contents to a new vial before reinjection (4a-nv and 4b-nv). I am considering all 4a samples to be equivalent and 4b samples to be equivalent. You can see from the data at the bottom of the table that the variability ( $\leq 0.5\%$ ) is approximately the same as it is for the between-sample variability for the data of Table 1 (0.5%). This reinforces the conclusion I drew in the previous paragraph that the injection process is working properly.

Table 2: Data for multiple injections of samples 4a and 4b.			
Sample*	% Assay	Sample*	% Assay
4a	86.11	4b	87.52
4a-ri	86.33	4b-ri	86.67
4a-nv	85.98	4b-nv	86.87
Average	86.14	Average	87.02
SD	0.18	SD	0.44
%RSD	0.2%	%RSD	0.5%
*a and b are original values from Table 1; ri is a reinjection of the original sample from the same vial; nv is a reinjection of the original sample after it was transferred to a new vial.			

Table 2: Data for multiple injections of samples 4a and 4b.

### What Is at Fault?

At this point, we've observed that sample 4 exceeded the maximum allowable difference between subsamples and confirmed that the difference between subsample 4a and 4b is indeed an outlier using the Dixon's Q-test. We have also shown that the results for both samples 4a and 4b have the same level of precision as the remaining samples, so it appears that the problem is not related to the injector. Let's see if we can further narrow the source of the problem to the primary sample 4 or one of the subsamples 4a or 4b. We have enough data now that we can compare the assay values and see if they are consistent.

First let's compare sample 4a and 4b. With the three "equivalent"

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value of  $t = 3.19$ , for a probability  $\alpha = 0.05$ , the critical value is  $t = 2.78$ , so the Student's  $t$ -test shows that there is indeed a significant difference between the mean assay values of sample 4a and 4b. The Excel report (not shown) refines this a bit and indicates that there is only a 3.3% chance that there is not a significant difference in means.

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Now we know that samples 4a and 4b are not equivalent. Can we extend the process further and decide if one or both of them are likely to have an error in assay value? We can do the same Student's  $t$ -test for sample 4a and sample 4b compared to the remaining samples. One might argue that this is stretching the test a bit, because the larger data set compares variation between samples, whereas 4a and 4b test within-sample variation, but let's ignore that for the moment and see what we get. First, we'll take the data from Table 1 and remove the injections for 4a and 4b, leaving 22 data points. Then we'll take the three data points for 4a and run the  $t$ -test comparison, then repeat it for 4b.

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When we compare the larger data set to sample 4a, we get a test value of  $t = 0.76$ , whereas the critical value is  $t = 2.07$ . This tells us that there is no statistical difference between the mean assay value for sample 4a and that of the remaining samples. With sample 4b, the test value of  $t = 3.20$ , which exceeds the critical value, means we can conclude that there is a significant difference between the mean assay value for sample 4b and the remaining samples. We may get a better concept of this if we view the data in Figure 3. In Figure 3, I have binned all the assay values from Table 1 into 0.25% increments. As expected, the general form is that of a Gaussian distribution, which would be the case for a large number of points containing random error. The mean (bottom of Table 1) is 86.34, which falls in the 86.5 bin. The value for 4a (86.11) falls in the 86.25 bin, which confirms what we found above: sample 4a is not significantly different than the mean of the remaining 22 values. The value for 4b (87.52), however, falls in the 87.5 bin at the extreme right of Figure 3. With a standard deviation (SD) of 0.42 for the data of Table 1 this means that 4b is 2.8 SD from the mean; for a Gaussian distribution, 99.4% of the values will fall within  $\pm 2.8$  SD of the mean. Contrast this to the smallest value of Table 1 (85.67), which is 1.6 SD below the mean; 89% of values should fall within  $\pm 1.6$  SD of the mean, so it is much less likely that 85.67 is an outlier than is 87.52.

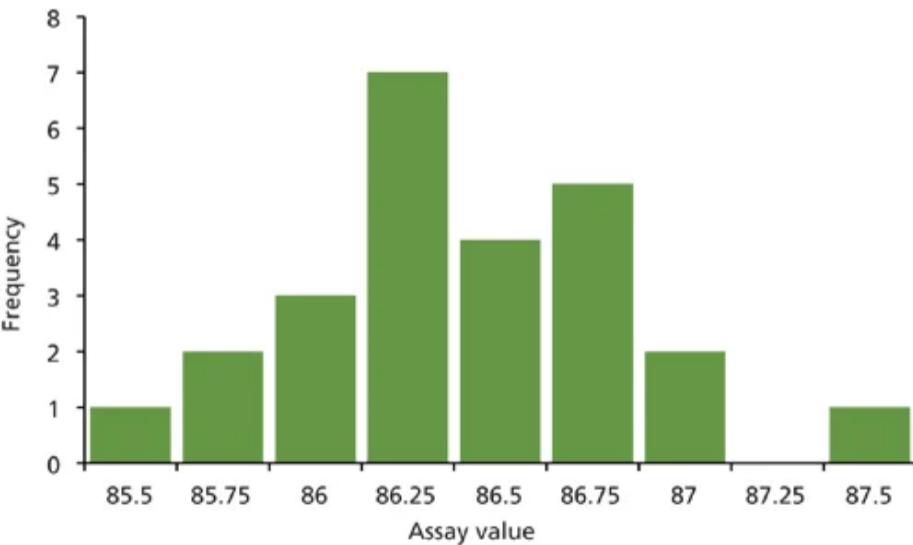


Figure 3: Frequency distribution of assay values for all samples from Table 1, binned into 0.25% increments.

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can we do to track down the source of the problem? First, we should eliminate the simple and obvious possibilities. The ones that come to my mind are transcription errors and integration errors. If the analytical balance has a printer attached, check the printer tape to make sure that the weight for sample 4b was transcribed correctly into the calculation of the assay value. For example, the reported weight (not shown) was 100.29 mg; if the decimal values were reversed in transcription from a true value of 100.92, the correct weight (100.92 mg) would change the 87.52 assay value to 86.92, and the difference between 4a and 4b would drop from 1.41% to 0.86%, and would pass the maximum difference test (1.0%) and bring sample 4b within 1.5 SD of the mean. Another possible error is in integration of the peak; double-check that the baseline was drawn properly.

At this point, you may feel that the investigation is complete. We've identified sample 4b as an outlier, and its companion 4a gives a reasonable value. Depending on your laboratory's standard operating procedures (SOPs), you may be able to drop 4b from the data set and use the data from 4a for reporting purposes. Write up your investigation report and you are done. Of course, you should keep your eyes open for similar failures in the future to determine if there is a high enough frequency of failure to merit further investigation.

If you want to investigate further, you need to consider all the possible sources of variation and determine if they are potentially important and if they can be reduced in magnitude. If the sources are independent of each other, the overall coefficient of variation (CV = %RSD/100) can be determined as the square root of the sum of squares of each variable:

$$CV = (CV_1^2 + CV_2^2 + \dots + CV_n^2)^{0.5} \quad [2]$$

where the subscripts represent each independent operation. The operations that come to mind in this instance are sampling, weighing, dilution, mixing, filtration, injection, and integration; there are probably others I've overlooked. Each of these will contribute uncertainty to the overall measurement. Sampling errors reflect how representative the subsamples are. For example, if the sample were granular sugar or a cup of coffee, the primary sample is very homogeneous, so taking a random sample should be fairly representative of the whole. On the other hand, if the sample were a bag of M&M candies, the distribution of the different colours in a small subsample would likely have much more variation. Thus, the homogeneity of the sample and the ability to take a representative sample would influence the sampling step. The variation in weighing could be tested by weighing a fixed standard weight multiple times. The reader did not specify how dilution was done; however, more uncertainty would be expected if a graduated cylinder were used to measure the liquid as compared to preparing the sample in a volumetric flask. Is the mixing sufficient for the concentrated sample and the diluent? Should mixing time be extended, agitation or sonication increased, or other variations in the mixing process be changed to improve the homogeneity of the diluted sample? Does filtering the sample affect the final result? This possible effect could be checked by comparing centrifugation to filtering and seeing if the

Before embarking on a detailed investigation of the method CV, you should step back and consider if it is likely that you will really improve the results of the analysis. A basic principle of statistics tells us that for independent errors, as in equation 2, the largest error will have the most influence on the overall error, that the overall error will never be smaller than the error of the largest contribution, and that the overall error will usually fall between the value of the largest error and twice that value. We determined in Table 1 that the overall method %RSD was 0.5% based on single injections of multiple samples. An autosampler that is operating well should have errors in the range of 0.3–0.5%, so it is unlikely that the overall error can be reduced much below the observed value of 0.5%. In other words, after a brief mental evaluation of the problem, I don't think I'd waste my time trying experiments to reduce the overall error. Instead, I'd stay alert to see if I could correlate future failures to some pattern in the analysis.

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## Conclusions

Let's review what we've been able to observe about the present problem:

- An error was found when the difference in assay values between equivalent subsamples exceeded the 1.0% threshold.
- By evaluating the difference between subsamples 4a and 4b both visually (Figure 1) and with the Dixon's *Q*-test, we showed that the difference was indeed an outlier from the remaining samples.
- We also concluded from the *Q*-test that the 1.0% threshold may have been a bit too tight because, based on the current data set, differences of 1.0–1.3% would fail the test criteria, but would not be proven outliers by the *Q*-test.
- Based on multiple injections of samples 4a and 4b, we used the Student's *t*-test to show that there was statistical difference between the mean assay values of the two samples, so they are not equivalent.
- We also used the *t*-test to find that the assay value for sample 4a fit within the normal range of the remaining samples, whereas sample 4b did not. This test correlated the cause of the problem with sample 4b, not 4a.
- We confirmed the association of the problem with sample 4b by plotting a frequency distribution of the assay values in Figure 3. Sample 4b was clearly at the extreme edge of the plot, whereas the value for 4a was near the middle.
- Before concluding the investigation, it was suggested that we check for obvious errors in numeric transcription and peak integration.
- We mentally evaluated possible sources of uncertainty

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investigate the failure. We were able to assign the error to a single sample (4b) and demonstrate that its paired subsample (4a) behaved in the same manner as the remaining samples, so it may be possible to use its results to obtain reliable assay data.

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"LC Troubleshooting" Editor **John Dolan** has been writing "LC Troubleshooting" for *LCGC* for more than 30 years. One of the industry's most respected professionals, John is currently the Vice President of and a principal instructor for LC Resources in Lafayette, California, USA. He is also a member of *LCGC Asia Pacific's* editorial advisory board. To contact John directly please e-mail:

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# Event: 31st International Symposium on MicroScale Bioseparations

Published on: March 1, 2015

**LCGC Asia Pacific**

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### *31st International Symposium on MicroScale Bioseparations*

The **31st International Symposium on MicroScale Bioseparations (MSB 2015)** will be held from **26–29 April 2015** at the **Crowne Plaza Hotel, Harbour City, Pudong, Shanghai, P.R. China**. The symposium series was first established to support the field of high-performance capillary electrophoresis, but now covers all major microscale bioseparation techniques including capillary electrophoresis, nano-liquid chromatography, microfluidics, mass spectrometry, hyphenated detection methods, pharmaceutical analysis, biotechnology, clinical diagnostics, food and health, nanoparticles, and advanced detection and instrumentation.



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Around 70% of the planned programme will be built from submitted abstracts, where a blind peer review with an enhanced submission requirement has been developed to ensure a very high calibre of science being presented. A layer of confidentiality has been introduced to protect conversations and presentations to allow the

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special session as part of the finale of the symposium. Overall, it is the intent of this symposium to foster the exchange of ideas at the frontiers of separation science.

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Four short courses are scheduled before the conference on 26 April, including "Immunoaffinity Capillary Electrophoresis for use in Biotechnology Applications" presented by Prof. Norberto Guzman from New Jersey, USA; "Multidimensional HPLC" presented by Dr. Gerard Rozing from Karlsruhe, Germany; and "Microfluidic Devices in the Life Science: Basics and Applications" presented by Prof. Jörg P. Butter from the University of Copenhagen, Denmark. More details are available from the website.

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Current printer and laser technology has enabled a method termed print, cut, and laminate (PCL) fabrication that is capable of creating printable microfluidic devices in a simple, cost-effective, and rapid manner, using relatively commonplace office equipment. Professor James Landers will present a lecture discussing the theory and methodology associated with each of the steps, as well the material challenges associated with substrates and chemistries used in this method. This will be followed by a hands-on part of the workshop, where those involved will carry out PCL fabrication using the necessary materials and some standard microfluidic designs.

The historical city of Shanghai has been chosen for this meeting, which will provide excellent networking opportunities to perform scientific discussions. On the 28 April, the organizers have planned the MSB Gala Banquet that promises food, live music, and the opportunity to network. Registration for the event will be limited to 395 participants to establish a "smaller meeting" ambience with a focus on the vigorous scientific exchange between delegates.

**E-mail:** [msb2015@msb2015.org](mailto:msb2015@msb2015.org) **Website:** [msb2015.org](http://msb2015.org) **Co-Chairs:** Yukui Zhang, Pengyuan Yang, Norman Dovichi, and Amy Y. Guo

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# Translations Between Differing Liquid Chromatography Formats: Advantages, Principles, and Possible Pitfalls

Published on: March 1, 2015  
[Patrik Petersson](#), [Melvin R. Euerby](#), [Matthew A. James](#)  
LCGC Asia Pacific

LCGC Asia Pacific,



*The numerous advantages of translating gradient chromatographic methods between the differing formats of LC have been explored and discussed. While translations in principle obey well defined chromatographic theories, the authors investigate a number of potential pitfalls that may result in poor translations as exhibited by selectivity differences, changes in efficiency, and hence failure to meet resolution system suitability criteria.*

Volume 18 Number 1  
Pages 6-16

**The numerous advantages of translating gradient chromatographic methods between the differing formats of liquid chromatography (LC) have been explored and discussed. Although translations in principle obey well-defined chromatographic theories, the authors investigate a number of potential pitfalls that may result in poor translations as exhibited by selectivity differences, changes in efficiency, and hence failure to meet resolution system suitability criteria. The consequences of these pitfalls are examined and the regulatory implications of method translation are explored.**

As a result of the introduction of commercially available sub-2-µm porous particles (1), sub-2-µm, 3-µm, and 5-µm superficially porous (2,3) particles, and ultrahigh-pressure liquid chromatography (UHPLC) instrumentation (4,5) from 2004 onwards, there has been an increasing interest in the ability to perform accurate translations between different liquid chromatography (LC) formats. An example would be translating between 150 mm × 4.6 mm, 5-µm *d*<sub>p</sub> formats on standard high performance liquid chromatography (HPLC) systems and 50 mm × 2.1 mm, sub-2-µm formats on UHPLC systems while maintaining the same resolution. The findings of a recent survey of

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- Translations between differing formats of liquid chromatography (LC) obey well-defined theories.
- Typically translations work very well.
- There are potential pitfalls that result in changes in selectivity or poor efficiency, but these can in most cases be addressed.

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There are a plethora of reasons for this shift in LC format usage and purchase, all of which are based on sound chromatographic theory (5,7,8). From the extensive experience of the authors within the pharmaceutical industry, the major drivers for this shift appear to be increased productivity (that is, reduced analysis time) coupled with minimal loss of information quality or an increased quality of data with no loss of productivity.

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## Advantages and Drivers

**Increased Resolution:** Reduction of the packing material particle size by a factor of two (that is, substitution of 3–3.5  $\mu\text{m}$  particles by 1.7- $\mu\text{m}$  particles), while keeping other operation factors constant, should result in an increase of resolution of approximately 30–40%.

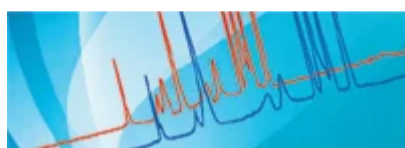


Photo Credit: traffic\_analyzer/Getty Images

**Speed of Analysis:** A reduction in column length ( $L$ ) and particle size ( $d_p$ ) while keeping the  $L/d_p$  ratio constant (for example, substitution of a 150-mm column with 3–3.5  $\mu\text{m}$  particles for a 75-mm column with 1.7- $\mu\text{m}$  particles) should maintain the same chromatographic efficiency and hence resolution, while reducing the gradient analysis time by 50% (same velocity typically used for large molecules) to 70% (higher velocity typically used for small molecules) and substantially increasing productivity. This approach is vitally important for the analysis of increasingly larger numbers of samples (that is, to better describe a process or formulation performance), increased utilization of instruments, the analysis of labile samples, and rapid at-line analysis (that is, process analytical technology).

A compromise between the approaches of increased resolution and speed of analysis has been the use of 100-mm columns with sub-2- $\mu\text{m}$  particles, which results in a 60% reduction in gradient time and an approximate 10% increase in resolution for small molecules.

**Reduced Solvent Consumption:** Converting a standard HPLC method that uses a 150 mm  $\times$  4.6 mm, 3–3.5  $\mu\text{m}$  column to a 100 mm  $\times$  2.1 mm, 1.7- $\mu\text{m}$  or 100 mm  $\times$  1.0 mm, 1.7- $\mu\text{m}$  column in theory offers a possible reduction in solvent consumption of approximately 86% to 97%. In practice, it is less often because of the necessity to prime the LC lines. During a global implementation of UHPLC within AstraZeneca (during the period of 2007–2010) involving 41 UHPLC systems, a reduction in solvent consumption of 63% was realized compared to the theoretical reduction of 77% (9).

**Ease of Method Transfer:** Within many industries it is often standard practice to transfer the chromatographic testing from the research and development (R&D) laboratory to a contract research

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in that QC laboratories, which have moved predominantly to on-line LC, may have to use UHPLC methods for the analysis of legacy products or methods that use HPLC columns.

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**Increased Instrument Utilization:** The drive for increased productivity and efficiency has necessitated an increased flexibility and utilization of available instrumentation. Many companies have a rolling programme to replace their worn out HPLC systems with a reduced number of UHPLC systems capable of running LC methods based on both 5-µm and sub-2-µm particles. In addition, valve arrangements are used that allow queuing of both HPLC and UHPLC methods on the same LC system, hence a reduced number of LC systems allow continuous operation.

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Principles of Method Translation

Because many translation guides successfully describe translations of isocratic LC methodologies (10), this article will focus entirely on the translation of gradient LC methodologies, which can be more difficult to perform. In theory, the translation required to maintain the chromatographic selectivity and performance of either an isocratic or gradient separation is very simple (7,11). The additional consideration that has to be accounted for in gradient separations is that a constant ratio must be maintained between the volume of each segment in the gradient over the column dead volume (8,12–15). After the introduction of sub-2-µm particles and UHPLC in the mid-2000s, several articles were published that focused exclusively on translations between 4.6- and 2.1-mm i.d. columns (7,8,10,16–18). To assist the practicing chromatographer in all types of translations, several commercial and academic computer applications have been developed (19); however, although these computer applications undoubtedly aid chromatographers, they all possess certain drawbacks to successful method translation.

In contrast to previous publications, this article only briefly describes the underlying theory and equations relating to chromatographic method translation principles (readers are encouraged to see the sidebar "Theory for Translations" for more information). Instead, this article will focus on how to perform successful translations of gradient chromatographic methods between HPLC and UHPLC systems, the accuracy that can be expected, and the potential pitfalls that chromatographers must be aware of and how to successfully avoid them. If the necessary precautions are taken, it is the experience of the authors that gradient translations work extremely well. For example, 11 LC methods were translated to or from UHPLC within AstraZeneca; the maximum deviations in relative retention times were only between 0.02 and 0.05, which was deemed acceptable (9,18).

**Theory for Translations**

In theory a volumetric translation of a chromatographic method is simply a matter of keeping the ratio between the volume of each segment in the gradient and the column dead volume constant

$$\frac{V_{g1}F_1}{V_m} = \frac{V_{g2}F_2}{V_m} \quad [1]$$

where subscript 1 corresponds to the method being translated, subscript 2 the translated method,  $V_{g1}$  the time for segment 1 in the gradient,  $F_1$  the flow rate, and  $V_m$  the column dead volume (12–15). The dead volume can be measured by injecting a dead time marker such as urea or thiourea to reversed-phase LC. This step is strongly recommended to get an accurate translation. The dead volume can also be estimated by the following approximation:

$$V_m \approx \left(\frac{d}{2}\right)^2 L_{TP} \quad [2]$$

where  $d$  is the internal diameter of the column,  $L$  the length of the column, and  $\kappa_{av}$  is the porosity of the column (typically ~0.6 for 100 Å porous particles).

In previous publications and software related to translations, it is assumed that the porosity of the columns is the same

and  $V_m$  in equation 1 can be replaced with  $\kappa_{av}V_p$ . However, when translating between a porous and a superficially porous particle it is unacceptable since the dead volume is reduced by the solid core of the latter. Differences in porosity will result in inaccurately scaled gradient times. For example, when translating from a porous particle to a superficially porous wide pore material the error in gradient time will be 36% (for the Aerie phase,  $d_p = 0.6 \mu\text{m}$  and  $d_{ps} = 3.2 \mu\text{m}$ ). Based on the knowledge of the diameter of the particle ( $d_p$ ) and the solid core ( $d_{sc}$ ) it is possible to estimate the porosity of the column packed with superficially porous particles.

$$\kappa_{av} = \kappa_{ps} + \kappa_{ps} \kappa_{sc} \quad [3]$$
$$\kappa_{sc} = 1 - \frac{\pi}{6} \quad [4]$$
$$\kappa_{ps} = \frac{\pi(d_p^3 - d_{sc}^3)}{6d_p^3} \quad [5]$$

where  $\kappa_{ps}$  and  $\kappa_{ps}$  are the fraction of the dead volume corresponding to inter- and intraparticle volume of mobile phase, respectively.

Since the porosity of columns packed with porous particles varies significantly, up to ~28% as described in the text, it is necessary to use experimentally

determined dead volumes to obtain accurate translations.

The flow rate is often scaled against column internal diameter to obtain the same linear velocity for columns with the same porosity.

$$F_1 = \frac{F_2 d_1^2}{d_2^2} \quad [6]$$

UHPLC is usually operated at higher linear velocities than HPLC because of the more favourable van Deemter curves. Thus, going from HPLC to UHPLC requires a flow higher than the scaled flow and vice versa. To translate to a flow rate scaled for another particle size, the reduced interstitial linear velocity ( $\lambda$ ) should be kept constant. This can be achieved by a scaling based on the following equation:

$$\Delta P_1 = \frac{d_p^2 F_1^2 \eta}{2g^2 L_{TP}^2} \frac{F_2}{F_1} \quad [7]$$

Translating flow rates, however, is difficult for superficially porous particles since both the A term and the C term in the van Deemter equation are smaller than for porous particles with the same diameter. In addition, it should not be assumed that the original method is running at an optimal flow rate. To make a translation to a truly optimal flow rate it is therefore recommended to translate

to a series of flow rates using equation 1 and thereby determine the optimal flow experimentally.

An approximate estimation of the maximal pressure for the translated method can be determined based on a pressure reading of the maximal pressure for the original method. Maximal pressure is obtained at approximately 20% (v/v) acetonitrile or 10% (v/v) methanol in water. It should be noted, however, that this estimate may be unreliable since both packing procedures and particle size distributions differ between columns and vendors (20). Furthermore, the system's contribution to the pressure also differs.

$$\Delta P_1 = \frac{d_p^2 F_1^2 \eta}{2g^2 L_{TP}^2} \frac{F_2}{F_1} \quad [8]$$

For an accurate scaling it is of critical importance that the ratio between dwell and dead volume is kept constant; that is, the difference in ratio between the system's dwell volume and the column dead volume needs to be negligible.

$$\frac{V_d}{V_m} = \frac{V_{d2}}{V_{m2}} = \Delta \quad [9]$$

where  $V_d$  is the system dwell volume and  $\Delta$  the difference in dwell and dead volume ratio between the systems. The dwell volume for an HPLC system

is typically 1–2 mL and for a UHPLC system it is 0.1–0.5 mL depending on configuration and model. For an accurate translation it is recommended to measure (22) the dwell volume (only necessary once for a certain instrument model and configuration).

If  $\Delta$  is positive, it is necessary to introduce an initial isocratic step of  $z$  min to the translated method corresponding to

$$z = \Delta \left( \frac{V_m}{F_2} \right) \quad [10]$$

If  $\Delta$  is negative, it is necessary to start the gradient  $z$  min before the sample is injected, a function available in certain chromatographic data systems. A delayed injection after the start of the gradient can be easily implemented on certain LC systems (such as the Waters Acquity H-class system or the Agilent 1290 system). Another alternative that partially solves the problem is to use a larger column internal diameter (equation 9).

If the translated method is not corrected for, it may display different relative retention times and potentially also different selectivity. The injection volume is scaled against the dead volume to obtain the same relative amount on column.

$$V_{in} = \frac{V_{in2}}{V_{m2}} \frac{V_m}{V_{m2}} \quad [11]$$

Because of the higher efficiency of sub-2-µm and superficially porous columns, asymmetry will be higher for overloaded peaks due to the higher concentration at peak apex. This can be compensated for by a scaling against the number of plates for a nonoverloaded peak chromatographed under isocratic conditions.

$$V_{in} = \frac{V_{in2}}{V_{m2}} \frac{N_1 V_m}{N_2 V_{m2}} \quad [12]$$

For porous particles the efficiency is proportional to the ratio of column length or particle size. Thus, for porous particles:

$$V_{in} = \frac{V_{in2}}{V_{m2}} \frac{d_p}{d_{p2}} \frac{V_m}{V_{m2}} \quad [13]$$

To minimise poor efficiency for early peaks it is important to match the column dead volume with the system's contribution to peak volume.

$$N = \left( \frac{V_m (1+k)}{V_{in} (k_{ps} + k_{ps})} \right) \quad [14]$$

where  $k$  is the retention factor and  $v$  is the contribution to the peak volume from band broadening in column ( $\kappa_{av}$ ) and system ( $\kappa_{av}$ ) expressed as standard deviation.

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LC systems of which the dwell volume and system volumes had been previously well characterized. Any experimental data including the injector programmes used can be supplied by the authors on request. For the delayed injection work on the Agilent LC systems, the injector must be in the bypass mode (that is, no flow through the injector). After the calculated delay time, the flow was returned to the mainpass position (that is, flow through the injector), then after a predefined time (time =  $[5 \times [\text{injection volume} + 5]]/\text{flow rate}$ ) to flush the sample onto the column, the flow was returned to the bypass position. At the top of the gradient the flow was returned to the mainpass position to wash the injector with the mobile phase to avoid carryover and subsequently flush the injector with the starting mobile phase composition prior to the next injection. Zorbax Eclipse XDB C18 columns (Agilent Technologies) of 1.8-, 3.5-, and 5- $\mu\text{m}$  particle sizes were selected for the small molecule work because they exhibit a good scalability with respect to efficiency and particle size ( $R^2 = 0.9$  for  $N$  versus  $1/d_p$ ) (20).

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Translations were made using a Microsoft Excel spreadsheet and the equations described in the sidebar "Theory for Translations." A corresponding software application for these types of translations is now freely accessible at the ACD/Labs website (21). In addition, a more complete set of translation tools is included in version 2014 of the ACD/Chrom Workbook software (22).

## Potential Pitfalls in Method Translations

### Observed Selectivity Anomalies as a Result of Differences in Dwell

**Volume:** The *system dwell volume* ( $V_D$ ) is defined as the volume from the point where the two solvents A and B first meet to the inlet of the column. For accurate translation of gradient methods it is of critical importance that the ratio between the dwell and dead volume ( $V_M$ ) is kept constant; that is, the difference in ratio between the system dwell volume and the column dead volume needs to be negligible ( $\Delta = [V_D/V_M]_{\text{original}} - [V_D/V_M]_{\text{new}}$  must approach 0). If this is not the case, then differences in chromatographic selectivity and relative retention time shifts may be observed. One such example is shown in Figure 1, where a series of analgesic related drugs have been chromatographed on the same 50 mm  $\times$  2.1 mm, 1.8- $\mu\text{m}$   $d_p$  column using a binary high-pressure mixing system with low dwell volume (that is, 202  $\mu\text{L}$ ) and a quaternary low-pressure mixing system with a higher dwell volume (that is, 990  $\mu\text{L}$ ). Note that in this example a translation from a system with  $V_D/V_M = 1.8$  (Figure 1[a]) to a system with  $V_D/V_M = 9$  (Figure 1[b]) resulted in coelution (peaks 7 and 8) as well as a reversal of elution order (peaks 3 and 4).

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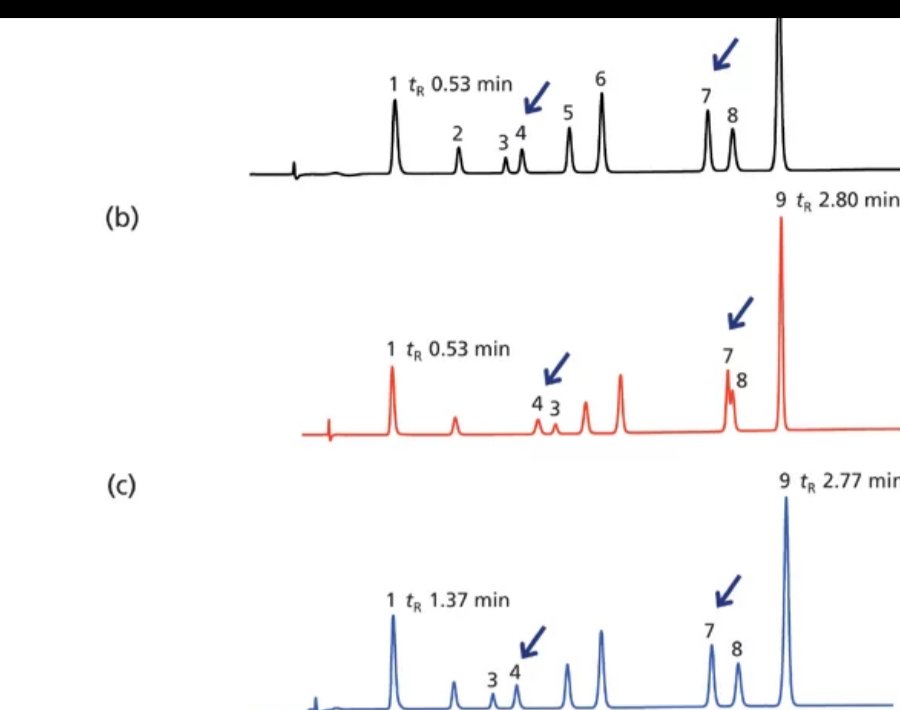


Figure 1: An illustration of how a larger  $V_D/V_M$  for the translated method can be compensated using a delayed sample injection ( $\Delta$  negative). The data also illustrates how  $V_D/V_M$  differences can affect the selectivity. The chromatograms have been scaled by alignment of the first and last eluted peaks. (a) binary model 1290 Agilent system,  $V_D/V_M = 1.8$ , injection in mainpass; (b) quaternary model 1290 Agilent system, no correction,  $V_D/V_M = 9.0$ , injection in mainpass; (c) quaternary model 1290 Agilent system, 0.93-min injection delay,  $V_D/V_M = 8.2$ , injection in bypass. Other conditions for (a)–(c): column: 50 mm  $\times$  2.1 mm, 1.8- $\mu$ m  $d_p$ ; flow rate: 0.75 mL/min; mobile-phase A = 0.1% formic acid in water; mobile-phase B = 0.1% formic acid in acetonitrile; gradient: 5–27% B in 2 min; temperature: 40 °C. Peaks: 1 = paracetamol, 2 = 4-hydroxybenzoic acid, 3 = caffeine, 4 = 3-hydroxybenzoic acid, 5 = salicylamide, 6 = acetanilide, 7 = aspirin, 8 = salicylic acid, 9 = phenacetin.

To compensate for a larger  $V_D/V_M$  on the translated method (that is,  $\Delta = [V_D/V_M]_{\text{original}} - [V_D/V_M]_{\text{new}} = \text{negative}$ ), the gradient must be started before injecting the sample - so-called injection delay or pre-injection volume - which can be easily implemented on certain LC systems (such as the Agilent 1290 or the Waters Acquity H-class systems). Equations 9 and 10 in the sidebar describe this process in detail. It should be noted that it is only necessary to determine the  $V_D$  (23) once for a certain LC configuration. Figure 1 illustrates how the injection delay principle can be used to compensate for  $V_D$  differences and thereby maintain the original selectivity and relative retention (Figure 1[a] versus 1[c]).

To compensate for a smaller  $V_D/V_M$  on the translated method (that is,  $\Delta = [V_D/V_M]_{\text{original}} - [V_D/V_M]_{\text{new}} = \text{positive}$ ) the translated gradient must possess an isocratic hold before commencing the gradient.

A small  $V_D/V_M$  results in a gradient with sharp changes (a Z-shaped gradient), whereas larger  $V_D/V_M$  will result in smoother, more S-shaped changes. This difference in gradient shape may also result in selectivity changes, mainly for fast separations on short columns (for example, 2-min gradients on 50 mm  $\times$  2.1 mm columns). To address this problem with possible selectivity changes, Agilent Technologies developed an approach (24) in which the company's model 1290 UHPLC systems can mimic the gradient shape of systems with a larger system dwell volume.

assume an equal porosity for all stationary phases irrespective of their nature (equation 2 in the sidebar). In cases where this assumption is not valid, significant errors in translated gradient times are possible. For example, large differences in porosity and hence dead volume can be expected and observed between porous and superficially porous particles (that is, 10–29% difference was observed for seven different types of superficially porous columns [25] and 13–37% predicted difference according to equations 2–5 in the sidebar). Consequently, translated gradient times may have an error of the same magnitude; for nonporous particles the error will be even greater (that is, 52%).

Equations 2–5 in the sidebar describe how  $V_M$  can be estimated for columns based on superficially porous particles using their reported particle and core diameters. However, these and other  $V_M$  estimates are all associated with an error that can be quite substantial. The use of quoted particle sizes may be misleading because there are many differing ways that particle size can be determined and reported (20). The pore size of the particles also affects the porosity (100-Å columns have a porosity of ~0.60, whereas 300-Å columns have a porosity of ~0.75).  $V_M$  estimations may also be inaccurate because of the fact that columns packed with different particle sizes are often packed with different pressures, which can result in lower porosities for columns designed for UHPLC. Consequently, for columns packed with porous particles, the difference between theoretical and measured dead volumes can be quite substantial. A comparison of 14 brands of modern columns from various vendors packed with porous particles exhibited a dead volume prediction error of  $\pm 28\%$  (25). This value corresponds to an error in gradient time of up to 28%. Figure 2 exemplifies how a 29% error in  $V_M$  can affect retention time as well as chromatographic selectivity.

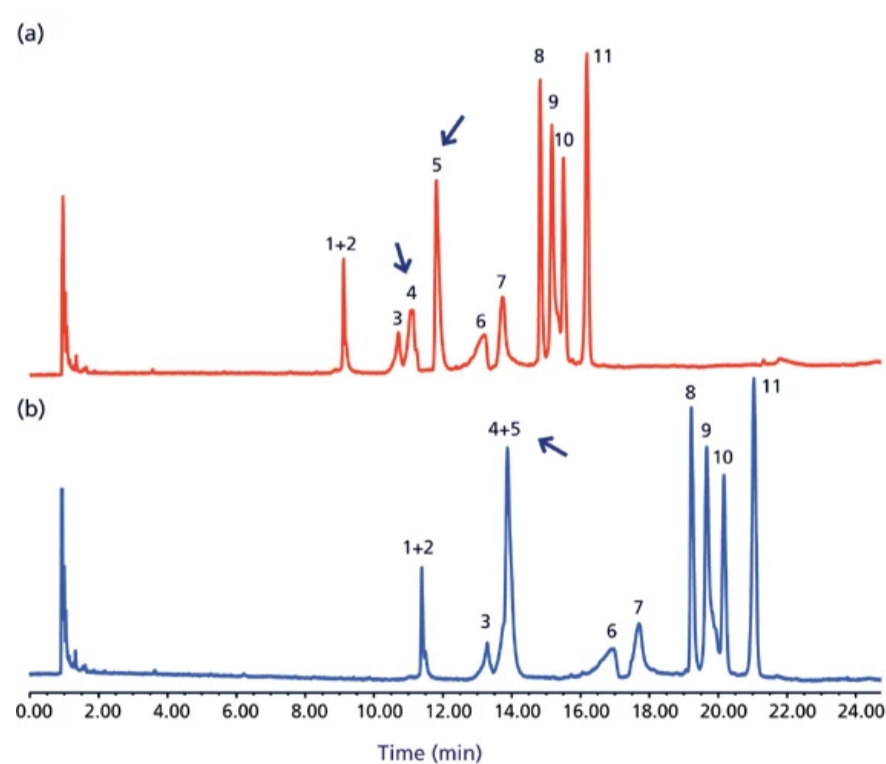


Figure 2: An illustration of how a translation from one column to another may affect both retention and selectivity if the translation is incorrectly performed (that is, assuming equal porosity between materials). Conditions: (a) Column: 150 mm  $\times$  2.1 mm, 3.6- $\mu$ m  $d_p$ , 3.2  $\mu$ m  $d_{core}$ ,  $V_M$  = 0.27 mL, 200-Å superficially porous particle Aeris widepore XB-C18; flow rate: 0.3 mL/min; gradient: 27–65% B in 30 min; mobile-phase A: 0.1% trifluoroacetic acid in water; mobile-phase B: 0.08% trifluoroacetic acid in acetonitrile; temperature: 40 °C.

$\mu\text{m}$  and  $0.2\ \mu\text{m}$ , respectively, with a measured  $V_M$  for the column of  $0.27\ \text{mL}$ . In comparison, when the methodology is translated using the same column (to eliminate any stationary-phase selectivity differences), but assuming that it is  $300\ \text{\AA}$  and fully porous in nature, the  $V_M$  is calculated as  $0.38\ \text{mL}$  and the new gradient yields a significant change in selectivity (see Figure 2[b]).

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Given the above discussion, the authors strongly recommend that  $V_M$  values are experimentally determined to obtain accurate translations.  $V_M$  can be easily determined by injecting a reversed-phase-LC dead time marker such as uracil or thiourea.

### Observed Selectivity and Peak Width Anomalies as a Result of Differences in Column Thermostat Design Between LC

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**Instrumentation:** Differences in column thermostat design used by differing LC instrument vendors will result in different temperatures being achieved within the column despite identical "set-point and feedback" temperatures being recorded in the column compartment. The extent of this temperature deviation is dependent on instrument design, flow rate, and temperature set point. According to our experience, deviations of  $5\ ^\circ\text{C}$  or more are commonplace (25). This degree of temperature difference is sufficient to cause significant selectivity differences when transferring a method from one type of LC instrument to another. Consequently, when translating from HPLC to UHPLC or transferring a method from one type of HPLC system to another, it is very likely that observed selectivity differences are related to differences in column temperature.

Column thermostat design affects not only selectivity, but also efficiency. It has been found that a precolumn heat exchanger in combination with a still air column compartment can reduce radial temperature gradients in the column and thereby results in narrower peaks compared to a water bath or a thermostat based on a forced air flow principle (26,27).

To address this problem, we recommend that the system suitability test (SST) section of the method describes how the resolution is affected by temperature and how corrections can be employed; this description can be achieved, for example, by illustrating chromatograms corresponding to  $30\ ^\circ\text{C}$ ,  $35\ ^\circ\text{C}$ , and  $40\ ^\circ\text{C}$  for a method developed on an instrument at  $35\ ^\circ\text{C}$  or by the construction of van 't Hoff plots (that is,  $\log k$  versus  $1/\text{temperature}$ ). By following this procedure, users can see in what direction and approximately how much the temperature needs to be changed to meet the SST acceptance criteria. This approach is supported by the United States, European, and Japanese pharmacopeias in that they allow a  $\pm 5\ ^\circ\text{C}$  adjustment of temperature to obtain the right selectivity (23,40,44).

**Observed Selectivity Anomalies as a Result of Differences in Heat of Friction:** When the mobile phase is depressurized in the column, frictional heat is generated, resulting in an axial temperature gradient. The heat generated is proportional to both the pressure drop over the column, but also the flow has an impact on heat generation and dissipation (28,29). On a  $2.1\text{-mm}$  i.d. column operated at a pressure close to  $1000\ \text{bar}$ , the difference between the column outlet and inlet temperature can be on the order of  $5\text{--}10\ ^\circ\text{C}$  for a  $150\text{-mm}$  column and  $10\text{--}18\ ^\circ\text{C}$  for a  $50\text{-mm}$  column (28,29).

Selectivity differences caused by heat of friction can be

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the observed change in selectivity is related to heat of friction and can be compensated for (18).

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### Observed Selectivity Anomalies as a Result of Differences in

**Pressure:** A change in pressure affects the molar volume of solvated analytes as well as their degree of ionization (30–34). This effect typically results in an increased retention as pressure increases; however, there are exceptions to this rule (34). Differing degrees of pressure-induced retention changes can result in either enhanced or reduced chromatographic resolution. It has been reported that proteins are more sensitive to pressure-induced retention effects than lower-molecular-weight analytes because their secondary and tertiary structures are additionally affected by pressure and flow (35).

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The majority of previous studies on pressure-induced retention effects have been conducted with a postcolumn restrictor to increase the pressure while maintaining the same flow rate and do not reflect typical chromatographic conditions. Furthermore, it has been suggested that heat of friction should counteract pressure-related retention and selectivity changes to some extent (28,35). Nevertheless, pressure-related selectivity differences are also seen during typical chromatographic conditions and for quite modest differences in pressure (for example, 142 bar) (36). Figure 3 illustrates a typical example of a translation from an HPLC method based on a 150 mm × 4.6 mm, 5-μm column (Figure 3[a]) to a UHPLC method based on a 50 mm × 2.1 mm, 1.8-μm column (Figure 3[c]) where a pressure increase of only 125 bar resulted in a significant change in selectivity (peaks 3 and 4). Unfortunately, it is difficult to compensate for these types of pressure effects. While it does not provide a solution, adding a postcolumn restrictor can enable confirmation that pressure is the cause of the problem (Figure 3[d]). To compensate for pressure-related selectivity changes, it is probably necessary to reoptimize the method by adjustments of parameters such as gradient shape and temperature.

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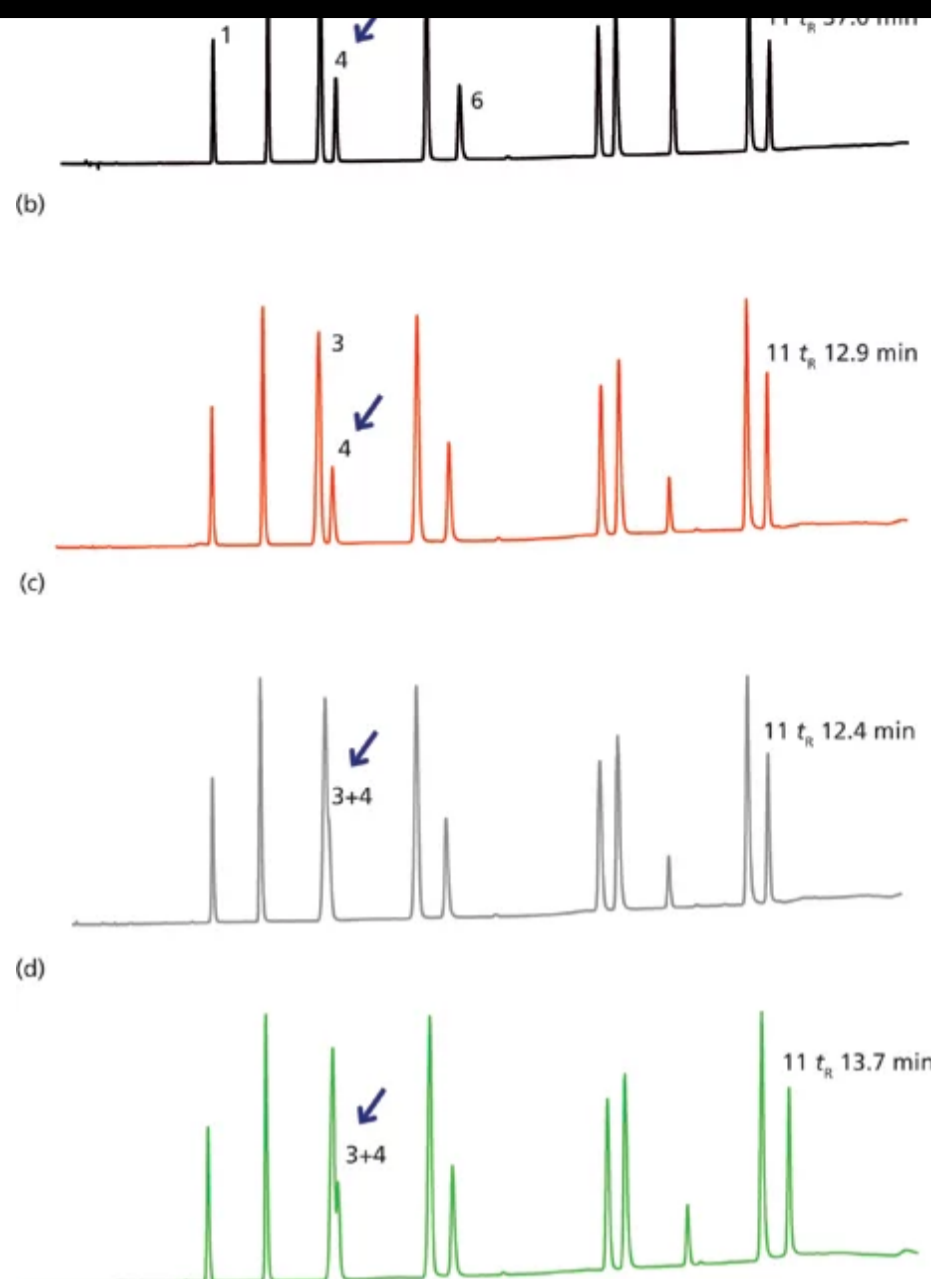


Figure 3: A translation while maintaining constant linear velocity from an HPLC method based on (a) a 150 mm  $\times$  4.6 mm, 5- $\mu$ m column to the corresponding UHPLC method based on a 50 mm  $\times$  2.1 mm  $\times$  1.8  $\mu$ m column (b) without and (c) with dwell volume compensation. The chromatograms have been scaled by alignment of the first and last eluted peaks. (a) binary Agilent 1100 system,  $V_D/V_M = 0.8$  (injection in mainpass), 103 bar; (b) binary Agilent 1290 system, no correction,  $V_D/V_M = 1.9$  (injection in mainpass), 231 bar; (c) binary Agilent 1290 system, 0.12 min injection delay,  $V_D/V_M = 1.0$  (injection in bypass), 228 bar; (d) as in (b) but with a postcolumn restrictor, 714 bar. Conditions (a): Flow rate: 1 mL/min; gradient: 5–100% B in 40 min; mobile-phase A: 20 mM monobasic potassium phosphate (pH 2.7) in water; mobile-phase B: 20 mM monobasic potassium phosphate (pH 2.7) in 65:35 (v/v) acetonitrile–water; temperature: 40 °C. Conditions for (b), (c), and (d) are the same as (a) except for a gradient time of 13.3 min, a flow of 0.208 mL/min. Peaks: 1 = terbutaline, 2 = N-acetylprocainamide, 3 = phenol, 4 = eserine, 5 = quinoxaline, 6 = quinine, 7 = ARD12495, 8 = diphenhydramine, 9 = carvediol, 10 = amitriptyline, 11 = reserpine.

#### Poor Efficiency Because of Differences in Extracolumn Band

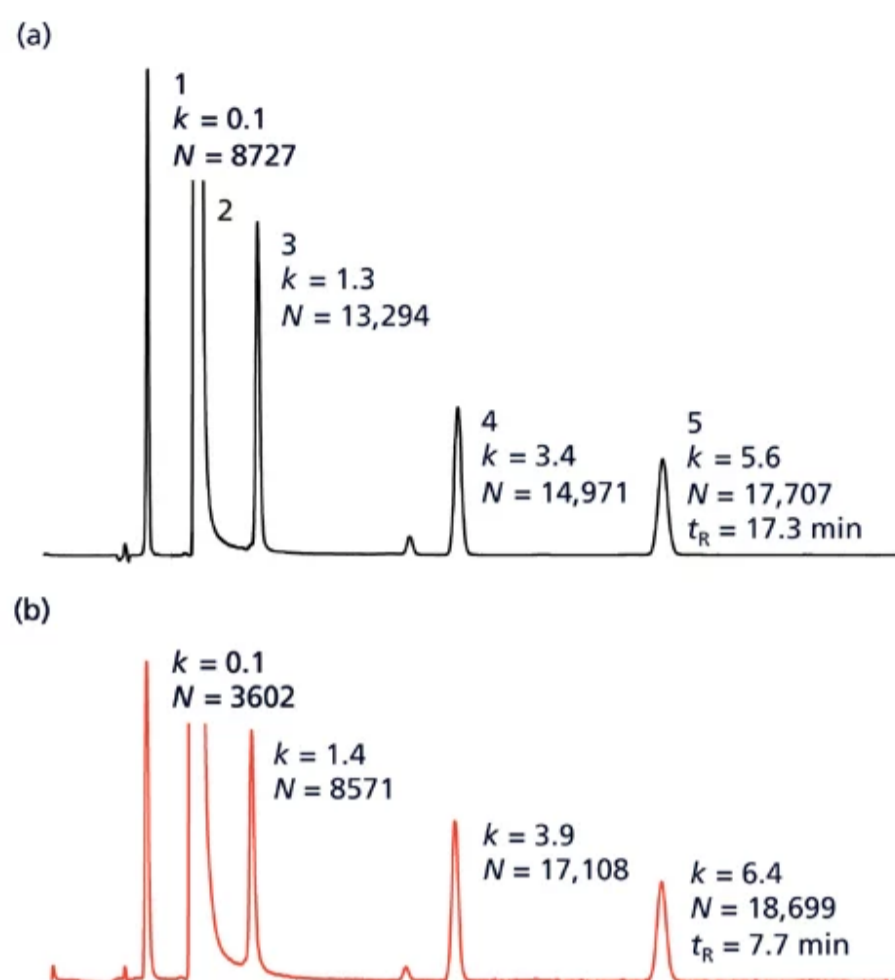
**Broadening Between LC Instrumentation:** Efficiency is dependent on retention as well as the ratio between column dead volume and the volume of the peak (equation 14 in the sidebar). Since the dead volume of an HPLC column is typically much larger than that of an UHPLC column, it is necessary to reduce the UHPLC instrument's contribution to peak volume significantly (7). This extracolumn band

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isocratic translation from HPLC to UHPLC exhibited the expected efficiency for late eluted peaks; however, the efficiency observed for early eluted peaks was significantly lower. Figure 5 illustrates the efficiency versus the retention factor for the same separation for other combinations of column and LC system ECBB volumes. In this example as well as in reference 7, the expected plate numbers are not reached until a retention factor of approximately 4 is reached when translating to a 50 mm × 2.1 mm column operated on an UHPLC system.

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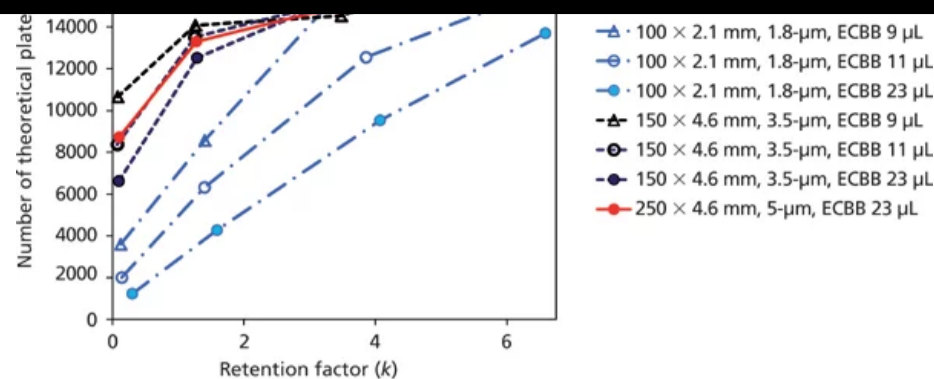
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Figure 4: An isocratic translation from (a) a 250 mm × 4.6 mm, 5-μm column and a binary Agilent 1100 HPLC system to (b) a 100 mm × 2.1 mm, 1.8-μm column and a quaternary Agilent 1290 UHPLC system, showing how ECBB becomes critical for peaks with low retention when using columns with small dead volumes. The chromatograms have been scaled by alignment of the first and last eluted peaks. Flow rate (a): 1.0 mL/min; flow rate (b): 0.21 mL/min; mobile phase: 2:400:600 (v/v/v) phosphoric acid 85% w/v–acetonitrile–water; temperature: 22 °C. Peaks: 1 = 4-hydroxybenzoic acid, 2 = acetylsalicylic acid, 3 = salicylic acid, 4 = acetylsalicylsalicylic acid, 5 = salsalate.

Modifying the ECBB of an LC system is usually quite difficult (38). A more realistic alternative is to increase the column internal diameter. Today, 3-mm i.d. UHPLC columns are commercially available, and they can significantly reduce this problem. The drawback to this approach is that a flow rate twice as high as for a 2.1-mm i.d. column must be used, resulting in a reduced saving in solvent consumption and a potential for increased heat of friction, which could cause band broadening and selectivity differences (29).

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Figure 5: Number of theoretical plates versus retention factor for the separations shown in Figure 4 and for five additional combinations of columns and LC systems with different ECBB contributions.

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In general, the minimized ECBB associated with UHPLC systems is advantageous; however, there is a possibility that it may cause significant band broadening when performing HPLC methods with the sample dissolved in a high proportion of organic solvent compared to the initial mobile phase. This may appear contradictory, but it can be explained by the narrower capillaries on the UHPLC system reducing the mixing between the sample and the mobile phase and thereby reducing peak focusing of the sample on top of the column. The solution to this problem is to reduce the sample volume or the amount of organic solvent in the sample solution. Alternatively, the capillary between the injection valve and the column can be replaced with a large internal diameter capillary to increase the mixing.

#### Differences in Linearity, Response, or Repeatability Related to

**Injector Design:** Different LC systems possess differing injection flow principles and materials in their autosampler construction. For example, in a loop injector the sample is typically exposed to larger surface areas and other materials than in a flow through needle injector design. This larger surface area may result in a more pronounced adsorption and, consequently, also a more pronounced nonlinear response at low concentrations for loop injectors.

Other potential problems can be related to differences in the internal diameter of the injector needle and capillaries. UHPLC systems have significantly narrower injector needle and capillary diameters, which can result in poor injection repeatability because of bubble formation if the draw speed is set too high in relation to the viscosity of the sample. Another related problem is that differences in viscosity between samples and standards because of matrix differences may result in differing amounts being injected, thereby the sample concentration can be under or over-estimated.

Consequently, it is important to validate linearity, response, and repeatability of the LC method when transferring a method from one type of LC system to another.

#### Differences in Peak Asymmetry Related to Differences in Efficiency:

The transfer of HPLC methodology to UHPLC when overloaded peaks are involved results in more-pronounced peak asymmetries being observed even if injection volumes have been correctly scaled against column volume. The explanation for this phenomenon is that

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the injection volume against column dead volumes as well as the isocratic efficiencies (equations 12 and 13 in the sidebar).

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**Other Issues:** In the early days of UHPLC, selectivity differences were commonly observed between columns of nominally the same material but differing particle size. These differences were impossible to explain by differences in the heat of friction or pressure differences (20). It was assumed that the base silica of the smaller particle size material was subtly different compared to its larger particle size counterparts. Today this difference is less of a problem. However, if a selectivity difference is observed that cannot be compensated for by either increasing or decreasing the temperature by a few degrees to mimic heat of friction or by adding a postcolumn restrictor to mimic a pressure-induced retention change, it is more than likely that it is related to subtle differences in the heterogeneity of the base silica used.

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## Regulatory Aspects

The degree of revalidation that is required for a translated method depends on the intended purpose for that method and where in the R&D process it is to be used.

For pharmacopeia methods, LC translations can, in principle, be made without any formal validation exercise being performed provided that the operating ranges described in the appropriate monographs (23,40,44) are not exceeded. Unfortunately, the current operating ranges limit the ability to translate from HPLC to UHPLC. However, the United States Pharmacopeia (40) recently opened up for translation of isocratic methods from 5- $\mu$ m porous particles to sub-2- $\mu$ m porous particles. Hopefully other pharmacopoeias will also follow this example and, in addition, allow translations of gradient methods as long as the specified selectivity and efficiency is maintained (that is, by maintaining or increasing the ratio between column length and particle diameter [41]).

For original pharmaceutical products it is necessary to validate all versions of the method that are used for analysis of samples to be used in toxicological or clinical studies. Do both the HPLC and the UHPLC methods require a full International Conference on Harmonisation (ICH) validation (42)? From a scientific point of view, it should be sufficient to submit a full ICH validation for either the HPLC or the UHPLC version of the method. For the other version, it should be sufficient to submit a minimalistic validation report claiming that the translation has been made according to first principles (that is, fundamental chromatographic theory). In principle, it should be enough to validate selectivity and linearity for the translated version of the method. It is advisable, at least during this transition period, to perform a full ICH validation and be prepared to submit the "missing" parts of the validation if requested by the authorities. It may also be prudent to include both the HPLC and UHPLC versions of the method in the experimental design used for validation of intermediate precision.

Postsubmission changes to methods are possible according to both the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) regulations. However, it is uncertain what is required in the rest of the world. In addition, the cost associated with a postapproval change is often considered prohibitive

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translating existing HPLC methodologies to those using newer column formats and UHPLC instrumentation can be realized. Advantages include increased resolution, enhanced speed of analysis, reduced solvent consumption, more efficient utilization of LC equipment, and ease of method transfer between HPLC and UHPLC. The authors describe a number of potential pitfalls that must be taken into consideration and avoided if accurate and reliable translations are to be achieved. These pitfalls include differences in LC instrumentation dwell volumes, which can lead to coelution of peaks and even reversal of elution order. The difference between the original and new  $V_D/V_M$  ratios must be accounted for: In the case of the new methodology having a larger  $V_D/V_M$ , a delayed injection must be used, or if it has a smaller  $V_D/V_M$ , an isocratic hold must be inserted before commencing the gradient. Errors in translated gradient times of up ~30% can be encountered unless experimentally determined  $V_M$  values are used. This may affect both retention and selectivity. Hence, the authors strongly recommend that chromatographers practically determine their  $V_M$  values. The effect of instrument differences between HPLC and UHPLC models have been highlighted as possible sources of translation error; these include differences in column thermostat design, the effect of extracolumn band broadening, and the influence of differing injector design. In addition, the effect of only moderately elevated pressures on selectivity has been demonstrated, coupled with the effect of increased heat of friction associated with smaller particles and the influence of increased efficiency on peak asymmetry has been described.

A free translation tool is available to assist the successful translation between HPLC and UHPLC methods (21). The tool is based on the principles described in this article and permits scaling of gradient times, flow rates, and injection volume as well as accounting for differences in  $V_D/V_M$  ratios between LC systems. A more comprehensive translation tool will also be available (22).

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