

# How to Improve Your Implementation of Two-Dimensional Preparative HPLC

## Solvent Viscosity Considerations

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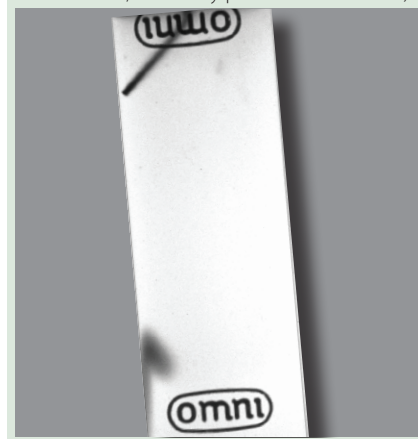
The biologics and natural product industries rely heavily on separation technology. Sample analyses are undertaken on the analytical scale, and isolation and purification are undertaken at the preparative scale. Key target components are often isolated to provide standard reference materials for future product quality assurance testing. These products are often very complex mixtures, requiring separation systems to have a high peak capacity for both analytical and preparative scale separations. A technique gaining popularity among companies that require the isolation of pure compounds from complex sample matrices is two-dimensional liquid chromatography, which can be conducted at either the analytical (2D-HPLC) or preparative

scale (2D-PHPLC). Operated in a heart-cutting mode, 2D-PHPLC is a technique for which the first dimension essentially serves as an extraction step, simplifying the sample matrix before separation of target analyte(s) in the second dimension. In the comprehensive mode (LC × LC), it is useful for analytical analysis and sample profiling.

Multidimensional chromatographic separation techniques use differences in the selectivity of each dimension that are oriented toward specific component attributes within a sample. Hence, different stationary and/or mobile phases are often used in each dimension. For example, common types of multidimensional systems include ion-exchange-reversed-phase, size-exclusion-reversed-phase, and (increasingly more useful) reversed-phase-reversed-phase. As such, a typical operational factor of a two-dimensional separation is that the mobile phase is different in each dimension, nearly always in at least the solvent composition, but often also in solvent type.

Coupled column multidimensional chromatographic separations require that a sample be transported from the first dimension to the second dimension as a dilute solution in a solvent plug. When operated on a preparative scale, this transport (heart-cut) volume fraction may be large to maximize sample

**Photo 1:** Photograph of a 10- $\mu$ L solution of iodine migrating along a chromatography column, following an injection at the wall (flow rate 1.5 mL/min; time of photograph 4.60 minutes postinjection; sample solvent carbon tetrachloride, mobile phase carbon tetrachloride, stationary phase YMC C18 silica)



recovery. Increasingly, scientists applying combinations of liquid chromatographic modes of separation are becoming aware of the limitations associated with solvent transportation processes, especially those that occur between two different separation dimensions. In particular, the phenomenon known as *viscous fingering* (VF) has been gaining increasing attention (1-17).

*Viscous fingering* is a flow instability phenomenon that occurs when two fluids with different viscosities come into contact with each other. When one fluid pushes the other, as is the case of a mobile phase and sample in chromatographic elution, the interface between these fluids can become unstable. The lower-viscosity fluid penetrates the higher-viscosity fluid in a complex interaction that resembles fingers. The phenomenon was first noticed by Hill in 1952 (1). But it was Saffman and Taylor who coined the name *viscous fingering* and investigated it (2). The first important paper detailing the significance of VF in liquid chromatography was published by Moore, who studied the effects of VF in size-exclusion chromatography (3). Numerous researchers have modeled the VF process and some have applied these models specifically toward chromatographic applications (4,5).

In the mid 1990s, Fernandez and coworkers (6-8) were the first to visualize

**PRODUCT FOCUS:** BIOLOGICS AND NATURAL PRODUCTS

**PROCESS FOCUS:** DOWNSTREAM PROCESSING

**WHO SHOULD READ:** MANUFACTURING, PROCESS DEVELOPMENT, AND ANALYTICAL PERSONNEL

**KEYWORDS:** COLUMN CHROMATOGRAPHY, SOLVENTS, PACKED BEDS, FLOW PROFILE, TWO-DIMENSIONAL SEPARATIONS

**LEVEL:** ADVANCED

the VF phenomenon in chromatographic columns when they used MRI imaging to view inside a column. These visualization experiments were closely followed by Broyles, Shalliker, and Guiochon, who visualized VF in glass-tube columns and used stationary and mobile phases that had exactly the same refractive indices (9). In this technique, an otherwise opaque resin bed becomes perfectly transparent. Colored solute markers injected into the columns were observed and photographed, recording the VF process. This provided absolute proof that viscous fingering was important in liquid chromatography.

Further studies by Shalliker and coworkers verified that significant changes in band shapes were apparent even when the viscosity contrast between the injection plug and the mobile phase was not significant enough to initiate a fully developed VF phenomenon (10–13). They coined this effect *previscous fingering* (11). These were important findings that highlight the need to pay particular attention to the viscosity differences between solute plugs (or heart-cut sections) and mobile phases, even when this difference is moderate and the actual VF process is not initiated — because the result may significantly change how multidimensional HPLC is undertaken, especially at the preparative level (14). How best to improve the performance of your multidimensional preparative HPLC separation (or at least how to minimize solvent-related inefficiencies) given that a viscosity contrast between solvents in each dimension may be a necessary factor in separation problems.

## EXPERIMENTAL

**Chemicals and Reagents:** We purchased LabScan high-performance liquid chromatography (HPLC) grade dichloromethane and toluene used for our VF experiments from Lomb Scientific Pty Ltd. in Sydney, NSW, Australia ([www.lomb.com.au](http://www.lomb.com.au)). We modified the solute plugs and mobile-phase viscosities by adding cyclohexanol from Ajax Chemicals Pty Ltd. of Sydney ([www.ajaxfinechem.com](http://www.ajaxfinechem.com)). VF was visualized with the use of oil red O dye purchased from Sigma in St. Louis, MO ([www.sigmaaldrich.com](http://www.sigmaaldrich.com)).

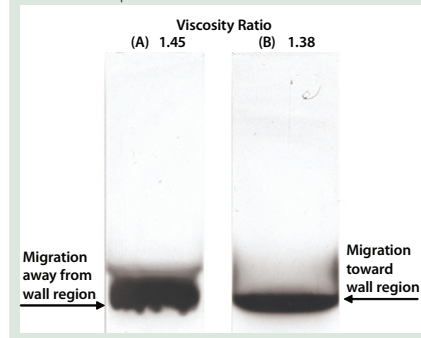
We purchased a Alltech nucleosil 10- $\mu\text{m}$  ODS(2) stationary phase to pack the glass columns from W. R. Grace and Co. of Deerfield, IL ([www.discoverysciences.com](http://www.discoverysciences.com)).

Under the experimental conditions we selected, the dye is not retained, so the injected band behaves as a tracer compound. We purchased reagent-grade carbon tetrachloride for visualizing the wall effect from Sigma. Care must be exercised when handling carbon tetrachloride due to its toxic and carcinogenic effects. So all work was performed in a well-ventilated hood, and protective precautions were taken as prescribed by the material safety data sheet (MSDS). We obtained iodine (99.9%) from General Chemical Division of New York, NY ([www.genchem.com](http://www.genchem.com)).

**Equipment:** For this visualization work, we used a model 6000 HPLC pump from Waters of Milford, MA ([www.waters.com](http://www.waters.com)) to deliver the mobile phase at prescribed flow rates as noted in the figure captions. Sample injection was achieved using a Valco VICI EHMA six-port, two-position switching valve with a 200- $\mu\text{L}$  injection loop ([www.vici.com](http://www.vici.com)).

The glass column was housed in a rectangular reservoir filled with dichloromethane to remove the cylindrical lens effect caused by the curvature of the glass column. The reservoir has four glass windows: Two are lit with fluorescent lights (15 watt), and one is used for “on-column” visualization and recorded using a Pentax ZXN SLR 35-mm camera fitted with a Tamaron 90-mm macro lens. For all experiments, a shutter speed of 125 and aperture of 8 were used, with Kodak professional PORTRA 160VC film. It was developed by a local commercial photographic processor. The remaining window could be looked through directly to observe each solute plug as it passed along the column, and video was sometimes recorded. We used a GBC LC 1200 UV/VIS detector set at 575 nm for conventional postcolumn detection ([www.gbcs.com](http://www.gbcs.com)). Responses were collected on a Lawson Labs A/D converter set at 2 Hz ([www.lawsonlabs.com](http://www.lawsonlabs.com)).

**Figure 1:** These photographs illustrate viscous fingering when the viscosity of a solute injection plug is (A) less viscous than the mobile phase and (B) more viscous than the mobile phase.



Chromatographic columns used in this study were packed in a 17-mm i.d. borosilicate (Pyrex) glass tube from OMNI of Cambridge, UK with end fittings made from Delrin plastic by a workshop at the University of Tennessee. These fittings include a fixed-length outlet fitting and adjustable inlet fitting, which allow for the axial compression of the stationary-phase bed.

**Column Packing Procedure:** We slurry-packed our chromatographic columns in a downward configuration, with the empty column filled with dichloromethane as a displacement solvent. A slurry of the packing material in methanol was pushed into the column and consolidated by a steady stream of methanol at 15 mL/min. The bed was then subjected to axial compression by applying mechanically a piston stress equivalent to a pressure of 48 kg/cm<sup>2</sup>, with both ends of the column open to allow the free flow of solvent within the bed. The compression piston was then removed, and the adjustable inlet fitting was tightened into place. For sample injection in the vicinity of the wall, we used a modification of the central-point method (18). A needle was inserted in the inlet frit and then bent at an angle so that sample could be loaded at the desired radial location. The head fitting was then inserted into the bed with minimal disturbance to the column inlet.

**Image Analysis:** We have previously discussed image analysis in detail, and it is beyond the scope of the present discussion (9–13, 18).

**Measurement of the Solute Plug Solvent Mixture Viscosity:** We measured the viscosities for mixtures of solute plugs and solvents with

various compositions using an Ostwald viscometer at either 20 °C or 25 °C. All measurements were made in triplicate, and the viscometer was calibrated using toluene.

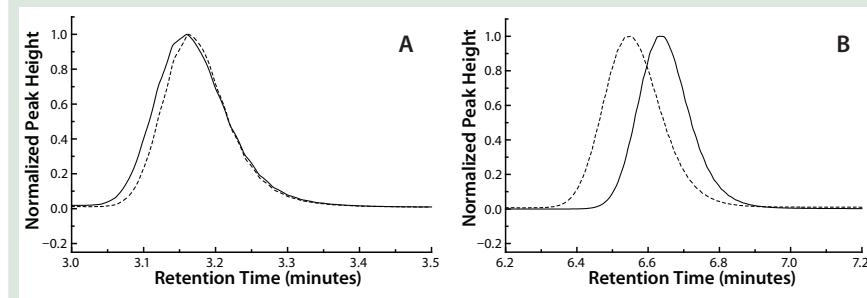
## RESULTS AND DISCUSSION

To date, almost all reports on VF in chromatographic elution observe the effect on unretained solutes. Only three papers detail the significance of VF under chromatographic conditions in which the solute is retained (14–16). In each case, serious band distortion was apparent. Had it occurred during a separation process, it would have led to failure of the attempted separation. The effects of VF on the band of an unretained solute are obvious. However, aside from the three papers noted above, very little information is available regarding the effect of VF on those of retained solutes. This ignorance must be addressed because almost all modes of separation use conditions in which the solute of interest is removed from the solvent plug. Before evaluating the effects of VF on a retained solute, it is important that we gain an understanding of the structure of a particle-packed chromatographic column.

**The Nature of Packed Beds: A** column packed with resin particles is typically made by consolidating those particles contained within a slurry, either by applying an axial dynamic stress or a high-velocity fluid-flow stream. In both cases, the resulting bed is not homogeneous. There is heterogeneity across the column radius, and along the column axis (18, 19). Although axial heterogeneity is important because it increases the reduced plate height, radial heterogeneity is the most significant source of limits in column performance because the band profile undergoes radial distortion rather than simply axial dispersion.

Across the radial column section, a particle-packed bed is more permeable in the central section, and packing density increases (so permeability decreases) toward the wall (18). However, the wall presents a special situation in its immediate vicinity. Particles cannot close-pack here because the wall and particles are both rigid. So

**Figure 2A:** Peak profiles of propylbenzene eluting in a 56/14/30 methanol/water/cyclohexanol mobile phase (injection volume 20  $\mu$ L, flow rate 0.8 mL/min; injection plug viscosities 80/20/0 [dashed line] and 56/14/30 [solid line] methanol/water/cyclohexanol)



**Figure 2B:** Peak profiles of propylbenzene eluting in an 80/20/0 methanol/water/cyclohexanol mobile phase (injection volume 20  $\mu$ L, flow rate 0.8 mL/min; injection plug viscosities 56/14/30 [dashed line] and 80/20/0 [solid line] methanol/water/cyclohexanol)

the wall does not bend to accommodate the particles, and the particles do not flex to pack tightly in this region. Hence, permeability rapidly increases as the void space increases because of lower packing density at the wall. The void space (and thus local bed permeability) are at their highest immediately adjacent to the wall. Solute that migrates in this region of the bed therefore does so with the highest velocity.

Photo 1 shows a solute band migrating along the wall of a column. In this photograph, the solute was injected directly into the column bed at a depth ~1 cm below the inlet frit and onto the wall. Details of these experiments were published in previous work, and further discussion here is not warranted. Suffice to say that migration in the immediate vicinity of the wall is ~40% faster than migration in the column center (18).

**Influence on Solvent Distribution in a Chromatography Column:** The higher void space along the wall region of a chromatography column is very important because it greatly influences the nature of solvent distribution under conditions whereby two solvents with different viscosities are in contact. Because the region along the wall has a higher permeability, flow resistance decreases. Hence, the higher viscosity fluid is preferentially displaced to this region of the bed.

That can be seen clearly in photographs illustrated in Figure 1, which shows the injection of a plug of solvent having a lower viscosity than the mobile phase (Figure 1A) and the injection of a solute plug with a higher viscosity than the mobile phase (Figure 1B). In both instances, the

ratios of solvent viscosities were similar. In Figure 1A, the solute plug migrates away from the wall region, as shown by the arrow. In Figure 1B, the solute plug migrates toward the wall region, as shown by solute trailing the main section of the band. This point cannot be sufficiently illustrated in two-dimensional print form. However, real-time observation of this band profile reveals that the elution profile shown in Figure 1B is cup-like: hollow in the center with sample trailing along the wall. Such behavior is apparent even when the viscosity contrast between the injection plug and the mobile phase is very small and insufficient to cause VF (11, 12).

An important aspect of the findings depicted in Figure 1 is that different wall-elution behaviors take place depending on whether solutes enter a chromatography column as solutions in a solvent that is either more or less viscous than is the mobile phase. The consequences of these differences with respect to retained solutes are discussed below.

### How Viscosity Contrast Between Solute Injection Plug and Mobile Phase Affects Retained Bands:

It is extremely difficult to maintain the refractive index match between a C18 silica stationary phase and a mobile phase while at the same time varying both solvent viscosity and elution strength to achieve solute retention and maintain visual clarity. As a consequence, the following results represent the retention behavior that occurred in reversed-phase HPLC under retention conditions, in conditions under which viscosity of a sample plug is different

from that of a mobile phase, and there was no match in the refractive indices of the two phases. Experiments were thus conducted in stainless-steel columns and the results evaluated using conventional postcolumn detection.

The chromatographic profiles illustrated in Figure 2 are that of propyl benzene. In Figure 2A (dashed line), the injection plug composition was methanol/water (80/20) and (solid line) (56/14/30) methanol/water/cyclohexanol. Both injections were made into a mobile phase of (56/14/30) methanol/water/cyclohexanol. The viscosity difference between the sample plug and the mobile phase was 0.811 cP in the case of the peak profile displayed by the dashed line, with the injection plug solvent being less viscous than the mobile phase. In the case of the solid-line profile, the difference in viscosity between injection plug and mobile phase was 0 cP. In Figure 2B, the mobile phase for both injections was (80/20) methanol/water, and the injection plug composition was (solid line, peak profile) (80/20) methanol/water and (dashed line, peak profile) (56/14/30) methanol/water/cyclohexanol mobile phase. The difference between viscosities of injection plug and mobile phase was 0.811 cP in the profile displayed by the dashed line, with the injection plug being more viscous than the mobile phase. In the solid-line profile, the difference in viscosity between the injection plug and the mobile phase was 0 cP.

In both examples depicted in Figure 2, where there was a 0.811 cP difference in the viscosity contrast between the mobile phase and the injection plug, there was no obvious indication on the chromatogram of any irregularity. The elution profiles are almost Gaussian, and there is no indication of peak splitting or shouldering (as is often the case when VF effects are significant but is consistent with the previscous fingering effect). However, when the elution profile is compared directly with that of a solute injected into the system under conditions in which the viscosity of the mobile phase is matched to that of the solute, differences become apparent (Table 1). These are as follows:

- When solutes are injected into a column in a solvent plug that is more

**Table 1:** Viscosity contrast between injection plugs and mobile phase

Viscosity Ratio (cP)	Propylbenzene (k)	Propylbenzene $\sigma^2$ (N)
Viscosity of Injection Plug More Viscous than the Mobile Phase		
0.000 (0.00)	2.80	5.980 <sup>3</sup> (7778)
0.811 (1.30)	2.63	9.924 <sup>3</sup> (4277)
Viscosity of Injection Plug Less Viscous than the Mobile Phase		
0.000 (1.00)	0.76	2.7956 <sup>3</sup> (3572)
-0.811 (0.64)	0.76	2.1470 <sup>3</sup> (4653)

viscous than the mobile phase, their band profiles exhibit fewer theoretical plates than they would in a matched-viscosity system.

- When solutes are injected into a column in a solvent plug that is more viscous than the mobile phase, their retention factors are smaller than they are in a matched-viscosity system.

- When solutes are injected into a column in a solvent plug that is less viscous than the mobile phase, their band profiles exhibit more theoretical plates than in a matched-viscosity system. In this case, no change was observed in the retention factor.

Table 1 details these differences. This effect becomes more substantial with increasing injection volume (20).

An obvious argument could be made that the addition of cyclohexanol to solvent in the injection plug actually caused the decreasing retention factor and number of theoretical plates — because cyclohexanol would increase the solvent strength. But this would be inconsistent with the experimental results. When the retention factor decreases (as viscosity contrast and solvent strength increases), band variance actually increases, which is inconsistent with the consequences of an effect being caused by an increased solvent strength because band variance should *decrease* along with elution time, not *increase* as is reported here.

Note also that Table 1 indicates that the column was less efficient at the same volumetric flow rate when the mobile phase viscosity was high, which is consistent with chromatographic behavior described above. It is therefore important not to compare results from each set (those at high and low viscosity of the injection plug) with each other, but rather to focus on the changes that take place within each set (the decrease of efficiency taking place when the injection plug is more viscous than the

mobile phase and its increase when the plug is less viscous).

**Explanation of This Effect:** For the wall effect and viscous fingering in particle-packed beds, the most likely explanation is obtained by considering a solute's path as it migrates along a column. When solute is dissolved in a solvent plug that is less viscous than the mobile phase into which it was injected, the VF phenomenon shown in Figure 1A indicate that the solute moves away from the wall region. By contrast, if solute is dissolved in a solvent plug that is more viscous than the mobile phase, it is moved preferentially towards the wall (Figure 1B).

Those effects occur almost immediately upon entry of the plug into the column (11, 12). Hence the solute, even if present within the solute plug for a very short period, has its trajectory through the column altered by the flow pattern caused by the viscosity contrast. Solute projected away from the wall can migrate along the column without the wall effect. But a plug of solvent directed toward the wall experiences a significant wall effect during its column residence time. It is well known that the wall effect is detrimental to column efficiency, with elution in an “infinite-diameter” column being the ideal state of solute migration.

**Best Use of the Separation Space — Solvent Viscosity Considerations:**

Chromatographers almost always prefer low-viscosity mobile phases over high-viscosity ones. This makes sense because it provides for higher column efficiency, lower inlet pressure, and increased separation speed. The limits of unidimensional separations are for a greater part related to limitations of inlet pressure (21). So in some ways, our results appear somewhat trivial. Who would deliberately use a high-viscosity mobile phase in the second dimension just to then heart-cut a lower-viscosity

solvent plug? This would not make sense if more efficiency were lost than gained.

Whereas chromatographers using unidimensional separations can for the most part control how their solutes are loaded, practitioners of multidimensional HPLC very often give little thought to the physical nature of the differences between mobile phases used in each dimension despite the fact that solute is transported from the first dimension to the second dispersed in a relatively large volume of solvent (compared with that of a conventional injection). These practitioners should be aware that the choice of which dimension is used in the first or second direction may be important — not only with respect to selectivity concerns, but also with respect to overall separation efficiency. To achieve maximum efficiency (and thus peak capacity), the first dimension should involve a mobile phase that is never more viscous than that used in the second dimension; otherwise, performance will be sacrificed and peak capacity lost.

This rule is even more important in preparative-scale separations because the viscous fingering effect becomes more substantial as both the injection volume (the heart-cut volume) and the column diameter increase (7, 20). Additionally, chromatographers should be made acutely aware that, if optimization is undertaken on an analytical scale with the intent of later scaling up to the preparative level, separation failure could result. The pre-viscous fingering effect at analytical scale scales up to full-fledged viscous fingering, which is related to column diameter, solvent injection plug volume, and solute concentration in the injected sample. Once fully developed, viscous fingering has been established, so it probably no longer matters which dimension has the most viscous mobile phase. The separation will perform poorly either way.

### A KEY CRITERION

The viscosities of the solute injection plug and of the mobile phase are important in unidimensional separations, but they are even more important in multidimensional separations, in which solutes are transported from the first dimension to the second in dilute

solutions (solvent plugs) that are usually larger than the injection plugs used in unidimensional systems.

When undertaking multidimensional separations, all care should be made to ensure that the solvent viscosity in both dimensions remains constant. In reality, however, that is almost impossible. As such, it is very important to prevent a marked reduction in separation performance by ensuring that the first dimension solvent is less viscous than the mobile phase in the second. Otherwise, separation performance will almost certainly be reduced. Relative solvent viscosities should therefore be a key criterion in designing two-dimensional separations.

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