Chromatography Today Help Desk

Developing Robust Chromatographic Methodologies

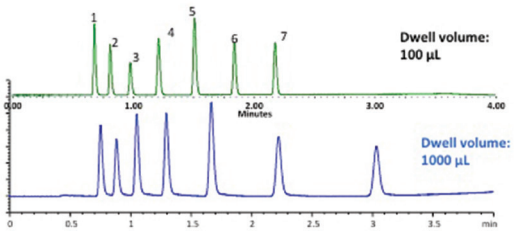
The world of chromatography is changing; laboratory scientists are moving away from being chromatographers to being chromatography users, and therefore there is a greater onus on the developers of chromatographic methods to make them more robust or at least have a greater understanding of what could cause them to fail. In this edition of Help Desk, we will discuss the implications of this for developing methods that will ultimately be transferred, either to different departments or to different organisations such as contract research organisations (CRO's) or contract manufacturing organisations (CMO's). Two issues will be highlighted, one of which has been previously discussed on the HelpDesk, both may seem trivial but both can have a significant effect on the performance of the assay.

When developing methods, chromatographers tend to focus on the immediate problem of being able to separate the various components of the sample. This can lead to a situation where the separation is optimised on a particular HPLC system, but if the assay is transferred to a different HPLC system it may actually change the retention times of individual components and as a consequence alter the elution order. This has obvious implications for assays that use detectors that are not discriminating, such as UV or ELSD, where the retention time is the primary descriptor for determining the analyte. As well as the effect a different pump can have it is also important to consider the effect of using other components that are different. In particular, the detector can have an effect on the integrity of the sample.

The HelpDesk was asked to be involved in developing a method for a customer. The method worked well and was validated and was used with real samples with no issues. Eventually the customer looked to outsource the method and this is where the problem started. The contractor received the method and came back to the client and stated that the method was not working, as there was a shift in the retention times of some of the components. Initially it was assumed that the contractor had purchased a column that was not working, and so proceeded to get a replacement which also suffered from the same fate.

So, the method went back to the original customer to check that the assay was working, and sure enough there was not a problem. As happens in this situation there then ensued an investigation into the method parameters to determine the stability of the assay, and during this phase it was determined that the isocratic portion of the method resulted in some movement of the compounds along the column which was affected by the delay volume of the pump. The original customer had used a very low dwell volume pumping system to develop the original separation, which the contractor did not have access to. Consequently, the contractor was using pumps which had a larger delay volume, which in turn affected the length of time of the gradient pump.

Figure 1



Separation Factors	Peak 1/2	Peak 2/3	Peak 3/4	Peak 4/5	Peak 5/6	Peak 6/7
100 µL	0.80	0.66	0.71	0.70	0.76	0.80
1000 µL	0.55	0.72	0.70	0.68	0.70	0.68

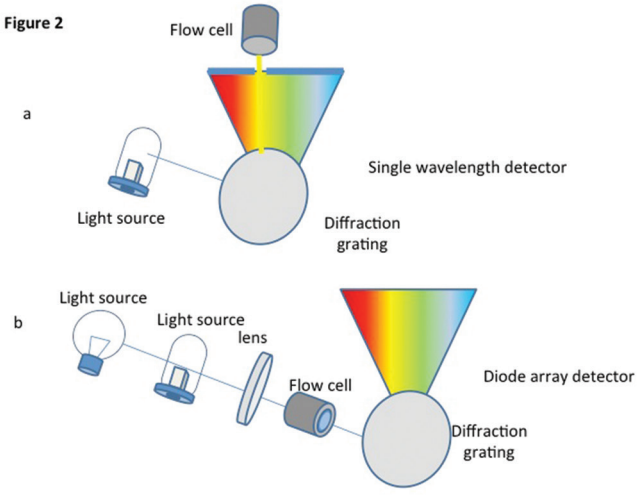
Figure 1 demonstrates the effect with the two systems clearly having different retention times for some of the peaks, looking at the earlier eluting compounds the effect is quite dramatic.

It is relatively simple to alter the effective dwell volume as a user goes from a large dwell volume system to a small one. This is achieved by simply holding the isocratic part of the separation for slightly longer to take account of the extra volume that the larger dwell volume system. There are a variety of approaches that can be used to determine the dwell volume and these will be covered in another help desk. So, for two systems, which have different dwell volumes, to give the same retention time the isocratic part of the separation will have to be modified, since the larger dwell volume causes the gradient to arrive later at the head of the column and the modifier concentration at the same retention time is lower when a higher dwell volume is present. In an isocratic system, all the retention times are altered uniformly, and so changes in elution order will not be observed.

If the user is taking a method from a low dwell volume system to a much larger one, and this can happen if a user is going from a UHPLC system to a lower pressure system which uses a proportioning valve to mix the solvents, such as for quaternary pumps, then the situation is substantially more complicated. The use of different starting conditions can be applied [1] but it is not guaranteed success due to differences in the elution times of different compounds at different mobile phase compositions.

Another aspect that has not been discussed previously on the HelpDesk is the effect that the detector can have on the assay. There are a range of different detectors that the chromatographer can employ, including UV (ultra-violet), MS (mass spectrometry), ELSD (evaporative light scattering detector), CAD (charged aerosol detector) and RI (refractive index). It should be noted that there are other detectors and that this list is not exhaustive.

The UV detector is the most common HPLC detector and is found in most laboratories, due to its economic value and its



applicability to a wide range of compounds. There are two formats that chromatographers use, one is a variable or fixed wavelength design and the other type is a diode array. The actual difference in terms of the operation is minimal, and the two types of detector are often used interchangeably. For the fixed wavelength detector, a UV/Vis light source is filtered to give the required wavelength, which results in the sample only being exposed to a single wavelength of light. With a diode array detection system, the sample is exposed to a full range of wavelengths, which are then captured by the array detector (Figure 2). This does not seem too significant, but for compounds which are sensitive to light this can be a significant difference resulting in the compound undergoing a change in the detector. If the compound changes in its form then the absorbance coefficient will also change which means the sensitivity of the assay will be affected. In the extreme example, the absorbance could be reduced by so much that the compound is no longer detected within the UV detector. The increase in biopharmaceutical compounds has highlighted this as an ever-increasing issue, since proteins can be very susceptible to degradation when exposed to a broad range of the electromagnetic spectrum.

The HelpDesk has discussed the importance of considering the effects of changing the instrumentation on the assay performance previously, and it is pertinent to reiterate this discussion. It is also important to consider that it is not just the pumps that can influence the performance of the assay and that the detector can also affect the very molecules that the chromatographer is trying to analyse. If changing the detector results in degradation of the analyte, then the sensitivity may alter, and in worse case situation complete loss of signal may be observed. As with all disciplines it is important not to make assumptions, and this is the case with assay development. It is important that the chromatographer understands how the analyte will be affected in each of the various stages of the analytical process to ensure that a robust assay is developed and that there are minimal issues when this assay is transferred.

References

1. L.R. Snyder, J.J. Kirkland, and J.W. Dolan, Introduction to Modern Liquid Chromatography, 3rd. ed. (Wiley, Hoboken, New Jersey, 2010)