Why is my Method Not Robust?

Chromatography Today Help Desk

With the first edition of Chromatography Today Helpdesk, it was thought one of the most common issues, namely of non-robust methods, should be addressed. Why do assays keep falling over and what can be done to make them stable is a question that keeps many scientists and lab managers awake at night. One of the biggest issues that users face though is that the assay has been validated and submitted to a variety of regulatory authorities across the globe, and the cost implication of revalidation becomes prohibitive.

So how is it possible that assays can become unstable, what needs to happen in the method development part of the process to ensure that the assay is more stable, and what are the key experimental parameters to monitor? There are different approaches that can be employed here, and the one that follows is a suggestion that can be used explicitly or used as a general guideline.

When an assay starts to fall over, information is the key to understanding what the real problem is. The information that is gathered should include;

- Data from previous sample batches analysed
- Data from the validation study
- Data from the method development study
- Physiochemical properties of the compounds being analysed (if known)
- An understanding of what parameters have changed

In terms of the latter comment, some care has to be taken when interpreting this information. Sometimes "Why was my assay working?" may be a more relevant question than "Why is my assay not working?" There is a natural assumption that if an assay has been working for some time and then fails, that the cause of the failure is due to the one parameter that is being changed, and whilst this is usually correct it is not always so. An important issue here is that some assays are just not stable, so relatively small changes, which may be out of the control of the analyst or supplier, result in the failure of the assay. This is very akin to the problem faced by Edward Lorenz [1,2] when he developed his atmospheric models and discovered that minute changes in the input parameters had a significant effect on the global weather system, the so called 'butterfly effect', Figure 1. In this case Lorenz observed that very small changes in the input parameters into a seemingly simple mathematical model for global weather, involving only three parameters, could have a dramatic effect on the final weather system. Lorenz stated that this was akin to a butterfly flapping its wings and causing a hurricane in another part of the world. The following problem exemplifies this scenario.

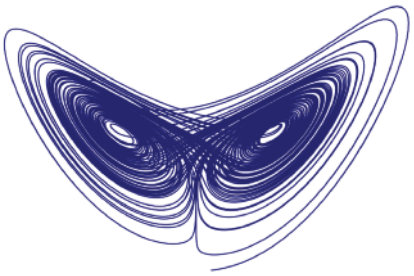


Figure 1: The classic solution to the Lorenz equation, highlighting that small difference in the input parameters can have a significant effect on the output data.

Figure 2 shows a real issue that separation scientists can experience. The original assay had been validated on one column and subsequently the same column had been used to run the initial set of samples. The

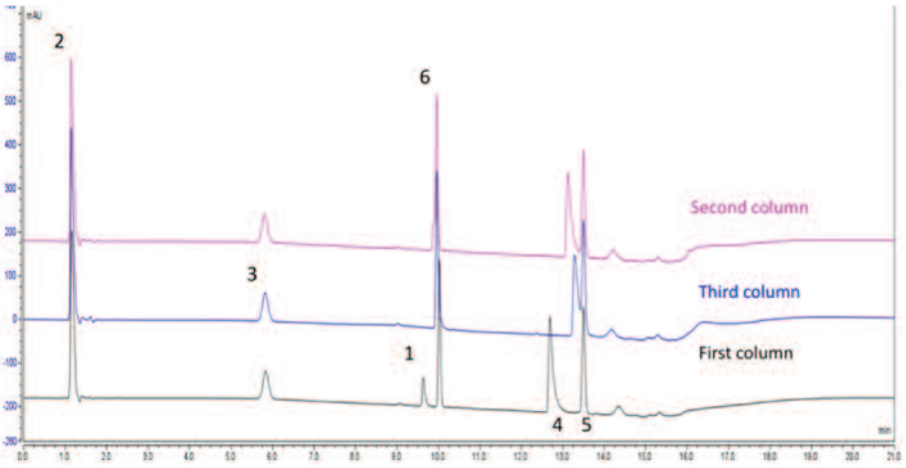


Figure 2: Stability of the assay is lost which can be seen through the lack of retention time stability for peaks 1 and 4 (doxylamine and doxepin)
Experimental conditions:
Mobile phase:A – 30 mM KH2PO4 (pH= 7), B – MeCN, Gradient : 10 to 80% B in 10 min, Flow rate: 0.2mL/min, Temperature: 30°C, UV Detection: 210nm, Injection volume: 5µL
Analytes:
1. Doxylamine (B), 2. Hydroxyisophthalic acid (A), 3. Benzamide (N), 4. Doxepin (B) 5. Flavone (N), 6. Fenoprofen (A)

column provided the required separation and was found to give very robust retention times. Eventually the column needed replacing and this is where the problems began. On receiving the new column the customer noted that there was a substantial loss in resolution for some of the peaks on the new column. It was thus thought that the issue was the column and so the customer tried another column only to find that the resolution provided by this column was of a similar quality to the second.

Clearly it must have been the column, as this is the only parameter that has changed, but on further investigation of the data and also of the experimental parameters another story starts to emerge. The first point to note is that the shift in the retention time only affects two components, all of the other peaks have a stable retention time, which suggests that the mechanism of retention for these two components is changing, but for the other components the mechanism is not changing. This is very unusual in chromatography, particularly in reversed phase chromatography where it is not common to have different primary mechanisms working on the same column. This has been known to happen when using some of the earlier silica columns, which due to their high metal content behaved almost like a mix mode phase [3-6], with the ability to have ion exchange in addition to hydrophobic interactions.

The two anomalous compounds suggested that a further investigation of the data was required so the physiochemical properties of the molecules under investigation were calculated [7], Figure 3. It was noted that the two problem compounds were both bases, with the neutral and acidic components not being affected by the change in the column. Investigation of the log D plot (the variation of hydrophobicity with pH) shown in Figure 3, highlights a potential flaw in the assay, since the pKa's of the two bases are very similar to the operating pH conditions. In subsequent CT Helpdesks the discussion will continue on the importance of log D and pKa in obtaining ideal chromatography. As a good method developer will know, there is a potential for some retention time instability at and around the pKa of a molecule. Many modern drugs will have several pKa's due to the number of ionisable groups that reside within the molecular structure; in fact there are rules that state for a compound to be a good drug it must have more than one ionisable group [8].

As a consequence of this investigation a new

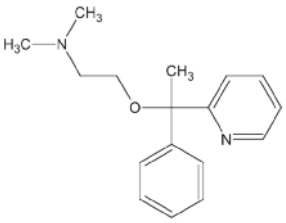
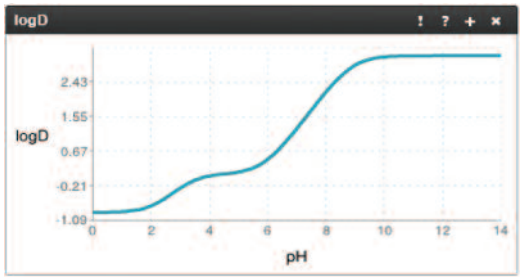
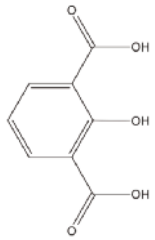
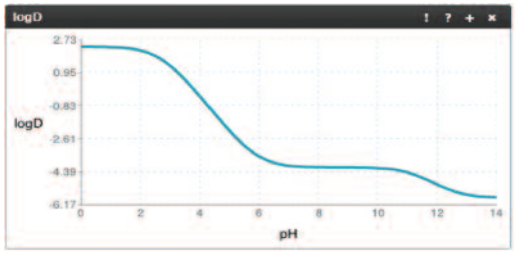
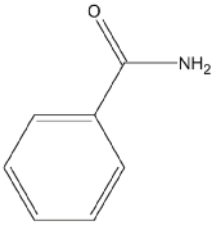
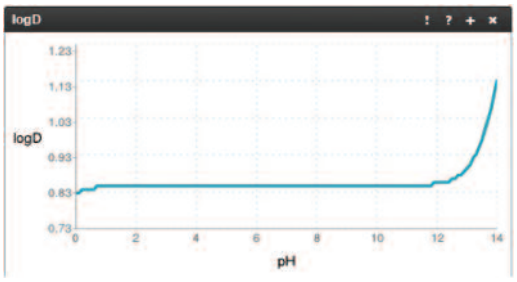
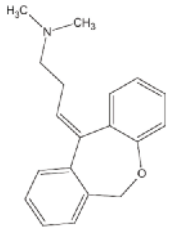
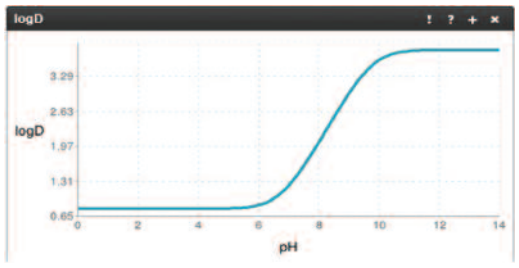
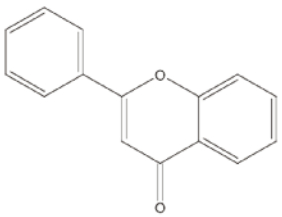
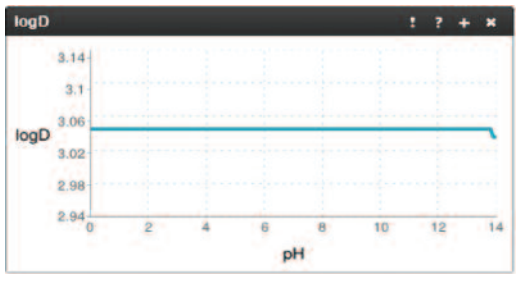
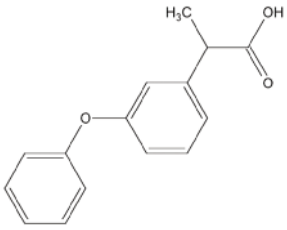
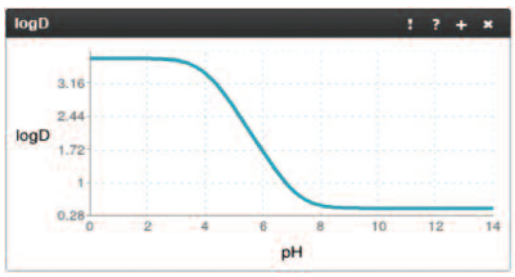
Cmpd #	Compound Name	Log D plot
1		
2		
3		
4		
5		
6		

Figure 3: Physiochemical data for the compounds being analysed, data obtained from [7].

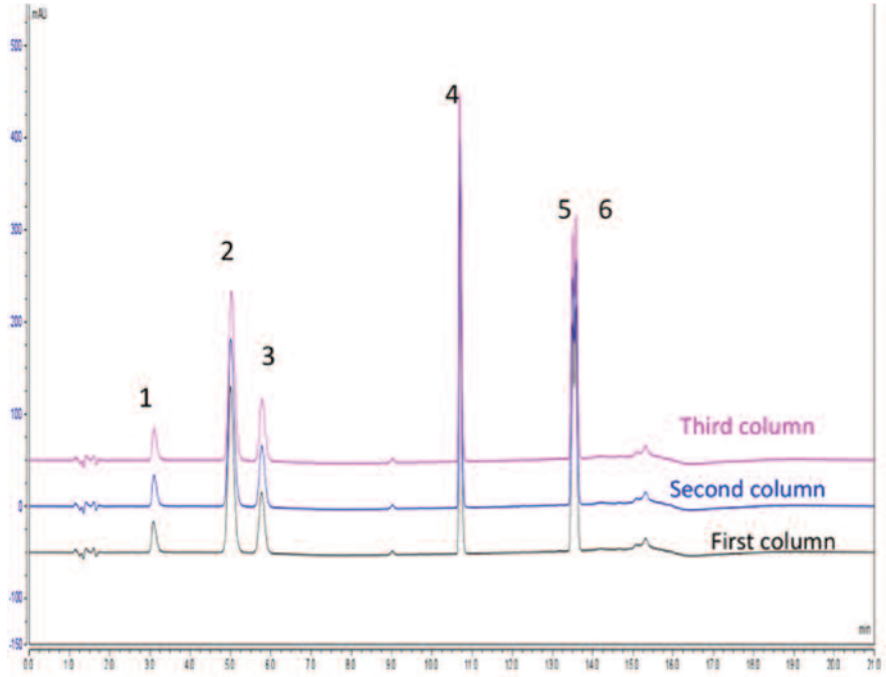


Figure 4: The new assay developed at low pH has a much greater stability.
Experimental conditions:
Mobile phase:A – 30 mM KH2PO4 (pH= 2), B – MeCN, Gradient : 10 to 80% B in 10 min, Flow rate: 0.2mL/min, Temperature: 30 °C, UV Detection: 210nm, Injection volume: 5µL
Analytes:
1. Doxylamine (B), 2. Hydroxyisophthalic acid (A), 3. Benzamide (N), 4. Doxepin (B) 5. Flavone (N), 6. Fenoprofen (A)

method was developed at a lower pH, Figure 4, and tested on three different columns from three different batches. The resulting chromatography needed further optimisation to separate all of the components but what the chromatogram shown which only differs from the original method in the pH that was used, does show is that the chromatography is stable from one column to another, and in particular the basic compounds which were causing the issue with the original method.

The use of predictive software [7, 9-12] can make method optimisation and assay stability determination a much less onerous task, virtually eliminating the need for lots of experimental data to ensure that the assay is stable. There are many commercially available products on the market, with most based on well understood theoretical models and for the majority of experimental arrangements these work incredibly well.

Conclusion

To avoid assay instability it is important that the chromatographic separation is stable to small changes that may not be within the control of the analytical scientist. Thus it is important to check the stability of the assay by varying a range of parameters for the final assay conditions. This should include:

- varying the temperature by ±5°C
- varying the pH by ±2 units
- using at least three different lots of column media and solvents
- checking to see what effect the dwell volume can have on the assay by running on different manufacturer's pumps.

The use of predictive software tools can substantially help here to reduce valuable laboratory time; however there is an initial cost implication to going down this route.

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