UHPLC – A Small Step for Man, or a Giant Leap for Mankind?

In the mid 1990's, HPLC was being described as a mature product. Most of the development was considered to have been done, and soon it would become a catalogue product like a hotplate stirrer. How far from reality that has turned out to be. HPLC is developing faster now than at any time since it was first introduced. Just think of the changes with mass spec, nano HPLC, high temperature HPLC, column developments, and by no means least, UHPLC.

At its outset in the early 1970's, HPLC used columns as long as 1 meter filled with 40-50um particles, and with injections made with a syringe through a septum directly into the packing bed at the top of the column. Then came microparticulate silicas, and 50cm followed by 25cm columns were introduced, packed with 10um materials. Separations were much better, but UV detectors were still spectrophotometers with a flow cell. Remember the old Cecil with a Waters M6000 pump? It was a 'new day in HPLC'.

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The issue with 10um packings was that they needed to be slurry-packed at high pressure, and the back pressure was reliant on a really narrow particle size distribution. However by the late 1970's we had 5um packings using irregular silica, with good efficiencies and a range of good bonded phases (microbondapak, partisil etc). By about 1980 we had spherical particles, offering a lower back pressure and which were much easier to pack. Suddenly a whole range of column packing companies arrived - some are still with us today. Thanks to them, the price of columns reduced from about £250 to nearer £100.

At this stage, HPLC looked very similar to the way it is today. Columns 15 or 25cm, flow rate 1ml/min, temperature ambient – 50oC, back pressure around 2000psi, with UV detection as the method of choice for most users.

The Need for Speed

We always need to analyse samples more quickly. Yesterday is never soon enough. Increasing regulation means more and more testing. Bringing a new drug to market requires thousands of tests on thousands of products and their metabolites. Clinicians want clinical results straightaway if possible. And we now have more work to do, with less people to do it.

The initial approach in the early 1980's was to use 3um packing materials. Smaller particle sizes give more efficient separations, and hence it was possible to use shorter columns and hence get shorter run times. As a rough guide, a time saving of about 50% was possible. Surprisingly though, 3um columns did not receive the rapturous response that might have been expected. One eminent scientist from the MRC who retired last year once told me that his run time had changed from 30 minutes to 12 minutes, but his sample preparation time had increased from 12 minutes to 30 minutes. His column cost had increased by 30%, and the column was much more sensitive to blocking up, and hence both his samples and mobile phase now needed filtering.

Another approach has been to use monolithic silicas. This is essentially a rod of highly porous silica, inserted into the column tube. Its high porosity allows the use of higher flow rates, which offers the faster analysis times. This is primarily the domain of Merck, and a number of applications are available from them. The speed improvement comes at a price, and columns are slightly more than double the price of conventional columns. Again, although they offer a speed increase, this technology has also not received the high market uptake that might have been expected.

High temperature is another option. It is still under development, but water has been found to act like methanol as an eluent at 200oC. The viscosity is much lower, and the problems mainly include finding columns, which are stable under hot water conditions, and getting the temperature down before the flow cell to avoid bubble formation. Cambridge Scientific is developing an FID detector for HPLC to overcome the need for this.

A number of suppliers are now offering fused core particles, otherwise known as core-shell particles or superficially porous particles. The centre of the particle is non porous and coated with a thin layer of highly porous material, giving virtually no band broadening and hence offering a highly efficient separation using a short column.

The final approach has been to use sub 2um particles, which has opened the door for uHPLC.

The appliance of science

When we increase the speed of analysis, either by reducing the column length or increasing flow rate, a loss in resolution occurs, so a prerequisite is to find a way to improve resolution.

$$\textit{Resolution}\quad Rs = \frac{1}{4}\frac{\{\alpha - 1\}}{\alpha}\frac{\{\kappa^{1}\}}{\{1 + \kappa^{1}\}}N^{0.5}$$

Assuming that we have already optimised selectivity we have only k' and efficiency N to work with. k' must be in the range 2-10, worst case 1-20, and this will have been optimised for the conventional HPLC separation. Below k' = 1, peak purity is questionable, resolution approaches zero, and integration is tricky. For k' over 20, there is virtually no gain in resolution because diffusion reduces the efficiency, so it is very important to keep k' within these limits.

This only leaves efficiency, N, as a means to improve resolution, and resolution is proportional to the square root of efficiency! ie if we double efficiency, resolution only increases by 1.4. It is also disproportionately expensive to improve efficiency, which is why when we develop methods we optimise efficiency last.

Efficiency can be increased by using a longer column, a smaller particle size, a lower viscosity eluent, a higher temperature, and by eliminating the causes of band broadening. Efficiency is the opposite of band broadening.

Causes of band broadening include:

Deep pores. Sample molecules spend part of their time in the stationary phase and part of their time in the mobile phase. More than 90% of the surface of the silica is inside the pores, and when in the pores, the sample moves slowly by diffusion. The deeper the pore, the longer that part of the sample remains stationary while the rest moves on in the mobile phase. The pore depth is controlled by the particle size, so the smaller the particle size the shallower the pores and the less band broadening occurs. Hence small particle size packings are more efficient.

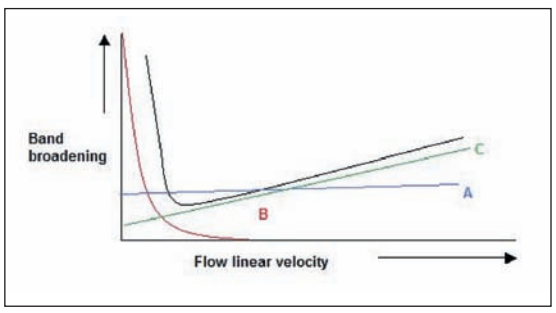
Diffusion. If allowed to do so, molecules diffuse in the column, causing band broadening. Diffusion is slow, and so only affects longer sample run times and slow flow rates.

Poorly packed columns. There are a myriad of pathways through the column. In order for all sample molecules to reach the other end in a tight efficient band, these pathways must all be similar in length and in resistance to flow. Essentially this is a measure of how well the column is packed.

Voids. These can occur in a column as a result of silica dissolution, or through settling of the packing material. Voids can also be present in the end fittings especially when tubing with the wrong ferrule lock distance is used, or in the tubing if narrow bore tubing is not used from the injector to the column and from the column to the detector. Voids allow the tiny injection volume to dilute, causing broader sample bands and lower mass sensitivity.

So in order to minimise band broadening, we must minimise all of the above. It is also pointless to invest in potentially expensive measures to improve efficiency without first ruthlessly minimising band broadening.

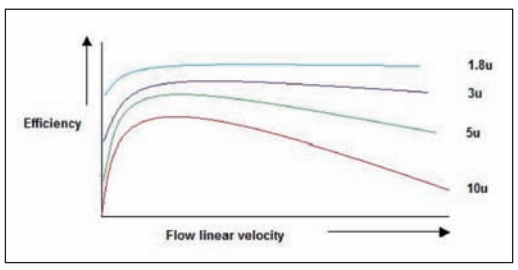
Band broadening is affected by flow rate, and this is represented by the van Deemter plot:



The A term (blue line) represents how well the column is packed. The B term (red line) represents the effect of axial diffusion, and the green line (C term) represents the effect of band broadening because of the depth of the pores. Summing these three together we arrive at the black line, which shows that band broadening is least at a certain flow rate, which for a 4.6mm id column equates to approximately 0.5ml/min.

Pumping too slowly gives rise to massive band broadening through diffusion, which is why it is so important to scale up the flow rate when working with prep and semi-prep columns. With an analytical column, increasing the flow from 0.5 to about 1ml/min is normal, because the drop in efficiency is small, but the run time is halved. Going further is possible, the limiting factor being the back pressure.

The significant discovery that leads to the development of UHPLC is the effect of particle size on the van Deemter equation. Plotting efficiency against flow rate (an inverted van Deemter plot) we see that it flattens out as the particle size decreases, becoming almost flat at 1.8um.



In the past it had not been possible to make particles at 1.8u, let alone to get a really narrow particle size distribution. But now particles can be made down at 100nm or smaller, and so the technology was there to make this a reality. The efficiency was a huge step forward, and the possibility existed to increase the flow rate with minimal loss in efficiency, thus providing a huge saving in analysis time. The problem was the back pressure. So HPLC systems were redesigned from the ground up to take much higher back pressures, and the result is the modern day uHPLC system, some of which can accommodate pressures up to 18,000psi.

What does UHPLC offer over conventional HPLC?

A significant reduction in analysis time.

How much of a gain is possible? Taking an extreme case where a method used a 25cm column on HPLC and could run on a 5cm column with uHPLC, then the speed gain from the reduction in column length is a factor of 5 (ie we save 80% of the original run time). Increasing the flow rate to say 3ml/min, would increase this by a further factor of 3, making a theoretical speed increase of a factor of 15. In reality, this is not achieved, but overall it may well be possible to analyse samples ten times faster than before.

An increase in mass sensitivity.

This is a real bonus. Because the injection volume is reduced and sample bands remain tight through the analysis, peak volumes are really small. This has an implication for autosampler design, flow cell design and data collection frequency, but it also gives us a significant improvement in mass sensitivity, typically a factor of 10.

An increase in the peak capacity.

Peak capacity is a measure of the separating power of a chromatographic system, and is generally defined as the total number of peaks, which can be resolved under isocratic conditions in a single chromatogram. Clearly the actual number depends upon the range of k' values which are used, the resolution of the peaks, and on the efficiency of the system. For an HPLC system with k' in the range of 0.5-20, N = 20,000 and Rs =2.0, Snyder et al give the Peak Capacity as 47. An advantage of UHPLC is that there is a significant increase in the value, in the range of 120 – 200 depending on the lab and the degree of system optimisation that has been carried out.

The main reason for selecting a uHPLC system is the speed advantage. Financially, this is the justification for the considerable addition cost of both buying and running a uHPLC system. But for users where sensitivity or the separation of large numbers of peaks is an issue, HPLC has now taken a huge step forwards and there may be no alternative to a uHPLC system.

What are the practical implications of changing to uHPLC?

Firstly a new UHPLC system must be purchased, which can cost almost double the cost of a regular HPLC system. The price comparison is perhaps a little skewed because the companies offering uHPLC equipment are those at the top end of the market, and therefore their standard HPLC products would have been the most expensive anyway. The new equipment will be designed to accommodate the high system back pressures, it will be able to handle small injection volumes very precisely, its detector will be set up to accommodate the smaller peak volumes, and its data system will be set up with high data collection frequencies, similar to those used for capillary GC.

Columns for uHPLC are typically 5cm long (although columns with shorter lengths are available for the simpler separations) and offering analysis times in less than 2 minutes. uHPLC columns typically cost about double the cost of a standard HPLC column, and require extreme care in use. Because the particle size is so small, the gaps between the particles are extremely small, and hence block up really easily. It is therefore recommended to filter samples and eluents using a very fine filter (0.2um) and to use fine sinters on the eluent lines to protect the column.

A further reduction in column lifetime arises because samples are processed so fast. If analysing ten times as many samples per day, it is clear that any physical or chemical contamination from the sample will block the column ten times faster. So column life can be a lot shorter than expected from experience with conventional HPLC, but this may not look so bad if the number of samples it can analyse measures the column life.

Not all column packings are robust enough to be produced at sub 2um particle sizes, and column packing requires considerable skill and care for very small particles. For this reason, some UHPLC columns perform very much better than others. It is also possible to encounter selectivity changes when converting a method to use uHPLC, which can mean that a method requires further optimisation, and in every case, it will require re-validation.

It would be normal to reduce the injection volume from 10-20ul to 2-3ul. The flow rate does not need to increase, but it may be desirable to increase the flow from 1 to maybe 3ml/min. Not all UHPLC systems can pump at over 2ml/min though, and those that can pump the higher flow rates cannot always then handle the really high back pressure, so it is worth checking before placing the order!

What does it cost to run? It is not surprising that a system operating at such high pressures will cost more for maintenance. Parts cost more and wear out more quickly. But for such a huge increase in sample throughput, it is not hard to justify the increased cost.

One problem area is the analysis of crude samples. Many labs have methods where a sample can be injected without sample preparation. For UHPLC, this must at least include filtration, but many labs have retained at least one HPLC system for this type of application.

So, is it a new day in HPLC, is it all change?

Without a shadow of a doubt it is a new day in HPLC. The capability of HPLC, in terms of speed of analysis, sensitivity and peak capacity, have all been improved by a huge amount, so this new technology is the best thing to happen to HPLC for a very long time. There have been many new developments, and many exciting possibilities are still in the pipeline, so this is an excellent time to be in HPLC! The future is certainly bright, if not orange!

Is it for everyone? This is most certainly the question. For a large lab with 30-40 HPLC systems, the economics of uHPLC is clear for all to see. A system which costs twice as much to buy and run is outstanding value if it does the work of 10 HPLC systems and maybe 2-3 analysts can do the work of 10. So its place at the top of the HPLC food chain is totally established.

Care should be taken to cover redundancy in uHPLC equipment. A lab running 30 HPLC systems can cope with an instrument failure relatively easily, but the same lab running 3-4 UHPLC systems might find it a little harder.

Is it appropriate for everyone? I think the only honest answer is no. For a lab which only analyses a small number of samples per day with maybe one HPLC system, then clearly the time saving would never pay for the increased cost of the equipment and the increased running costs. There is a cost/benefit analysis to be done to determine which is the most appropriate route to take.

If the benefit of increased mass sensitivity and/or peak capacity is of overriding importance, then regardless of sample capacity, UHPLC is the only choice. Otherwise the UHPLC platform has to justify its price tag by a reduction in running cost through increased sample capacity, or by increasing the workload of a given lab, especially important when today's multinational companies insist on buying each other till there's nobody left.

Since its inception, there has been a trend amongst the manufacturers who achieved so much by developing uHPLC and bringing it to market, to attempt to recover their development costs by selling it to everyone! The general approach is that the 'old HPLC' is for yesterday, and the future is uHPLC. The market has generally said no to this and as a result, a number of manufacturers are now introducing a lower spec and lower cost version of their uHPLC product, with a view to achieving acceptance by a wider customer base.

For those not wishing to junk their considerable investment in HPLC but requiring the higher performance now, the solution probably lies with a number of new columns based on fused core particles. These are typically 2.5um diameter and offer similarly high efficiencies to those of 1.8um particles in UHPLC columns, but without such high back pressure. It still means revalidating the method, but these columns can be used with conventional HPLC equipment to very good effect. Such columns are available from Phenomenex or Agilent, among others.

Having said all this, there is hardly a pump manufacturer in the world who is not redeveloping their standard pump to operate at a higher pressure. Excellent new injection valves were launched at Analytica by Sykam, which have a wear-resistant surface and can operate at higher pressures. So a gradual progress towards the use of higher pressure is likely. HPLC equipment has a lifetime of 10-20 years, and so many systems may yet be replaced by UHPLC.