# UHPLC – why all the hype?

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Over the last 25 years, HPLC has steadily developed with improved silica particles, bonded phases and narrower columns providing improvement in peak symmetry, selectivity and applicability. Whilst these incremental enhancements in column technology have improved the lot of the practising chromatographer, the promise of new technologies such as capillary electrophoresis, turbulent flow and monoliths have waxed and waned without major impact on main-stream HPLC separations. In some fields of analytical chemistry, such as bioanalysis, improvements in LC-MS/MS have relegated the chromatography system to being regarded as simply a means of introducing the sample into the ion source. Then one day back in 2005, an ex colleague now working for Waters, tried to interest HFL in a demonstration of the new Waters ACQUITY system. Frankly we were rather sceptical of the claims of the potential for improvements in speed and resolution of separations. The demonstration turned out to be more than impressive, as we took several of our existing validated LC-MS/MS methods and by applying UHPLC conditions were able to improve the run-time by around five times. As an added bonus, the sensitivity of MS detection was also improved by an average of 2 to 3 times. This was achieved by HFL scientists in a matter of days. The excitement in our labs was tangible as the potential impact that UHPLC could have on the business at HFL was discussed in terms of shorter run-times, greater instrument utilisation, enhanced chromatographic resolution and perhaps even the elimination of LC-MS/MS matrix effects, scourge of the bioanalyst. For us, this constituted the biggest step change in main-stream chromatography in the last 25 years.

## Applications

However, perhaps the best way to explain the benefits that UHPLC has brought is to give some examples. A major cost, and frequent cause of frustration, to any laboratory involved in research projects is the time taken to develop new analytical methods. The requirements of the assay in terms of the chromatography can differ substantially, depending upon the type of assay that is being developed.

In a bioanalytical laboratory, such as ours, where the emphasis is on plasma samples containing varying levels of new chemical entities the focus tends to be upon developing fast chromatographic conditions to obtain a high throughput of samples, with specificity being obtained from the use of selected reaction monitoring on a triple quadrupole mass spectrometer. Traditionally this has been obtained through the use of "ballistic gradients", i.e. the use of very fast gradients from low to high organic phase.

However, the use of UHPLC has demonstrated that it is possible to achieve even faster analyses, often without the need for ballistic gradients. An example of this is an assay we developed for Ciprofloxacin, an antibiotic that is used to treat conditions such as pneumonia and bronchitis. The aim was to develop a fast LC-MS methodology, suitable for quantifying low levels of ciprofloxacin in human plasma. Prior to the introduction of UHPLC to HFL's laboratories, a 4 min HPLC-MS method was developed using a short, cartridge based column (Phenomenex Mercury MS Luna 3 µm C18(2) 20 x 2.0 mm i.d.). An initial UHPLC method was generated, based upon the HPLC method, using the Waters ACQUITY UHPLC Columns Calculator software. Using this method as a starting point, within 30 min the analyst had optimised the conditions to those shown in Figure 1. It can be seen that even when injecting over three times less sample, UHPLC resulted in a doubling of the peak height. The analysis time was also reduced five-fold, from 4 min to 0.8 min.

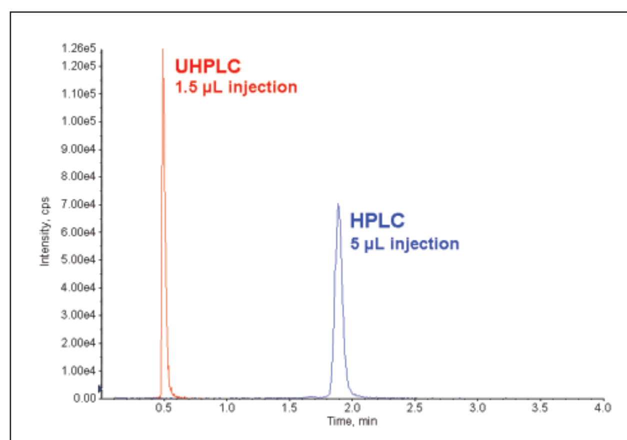


*Figure 1, SRM chromatograms for ciprofloxacin under HPLC (blue) and UHPLC (red) conditions*

Although the focus of a bioanalytical method is frequently speed and sensitivity, the presence of endogenous interferences, or matrix effects, can also require optimisation of chromatographic resolution. In this example we were asked to support a bioequivalence study, and to establish and validate an assay for ursodeoxycholic acid (UDCA), a naturally occurring bile acid found in small quantities in human plasma. When administered to patients, it is used successfully to treat the dissolution of gallstones as well as chronic liver disease. The particular challenge with this assay was that human plasma is known to contain isomeric, endogenous compounds which have to be chromatographically resolved from UDCA. The assay was initially developed and validated using a Waters ACQUITY BEH Shield RP18 column (50 x 2.1mm, 1.7 µm) in plasma samples sourced from volunteers in the UK. The chromatographic resolution of peaks looked adequate in UK control plasma because of the low concentration of the endogenous interference relative to the UDCA peak. However, the clinical study samples from Swedish volunteers contained much higher concentrations of the isomeric endogenous compounds, causing interference with UDCA (Figure 2). Attempts to increase the separation on the 50 x 2.1 mm column were unsuccessful as any improvement in resolution of the analyte from the interference was accompanied by deterioration in peak shape. As samples were awaiting analysis, a rapid solution had to be found to the problem. The simplest solution was to improve the resolution by increasing the number of theoretical plates, i.e. increasing the column length from 5 cm to 15 cm. The use of a longer column resulted in baseline resolution of UDCA from the interference peak (Figure 3) and only a modest increase in run time from 2 min to 5.5 min, still acceptable for a high-throughput bioanalytical assay.

Another example where chromatographic resolution and speed of analysis are primary concerns is in the simultaneous analysis of nine phytoestrogens in human urine and plasma. The application of UHPLC-MS has resulted in three major benefits to the assay. A reduction in analysis time was possible, from 14 min to 5.5 min by increasing the mobile flow rate. The time required for data processing was also significantly reduced as the software was able to automatically integrate the sharper analyte peaks produced by UHPLC, resulting in less user intervention. The enhanced UHPLC resolution allowed the inclusion of an analyte which could not be quantified using HPLC-MS. Figures 4 and 5 show the SRM trace for matairesinol under HPLC-MS and UHPLC-MS conditions respectively. Under HPLC-MS conditions, matairesinol co-eluted with a matrix interference from urine which made accurate quantification impossible. However, using UHPLC, the interference peak is baseline resolved from matairesinol.

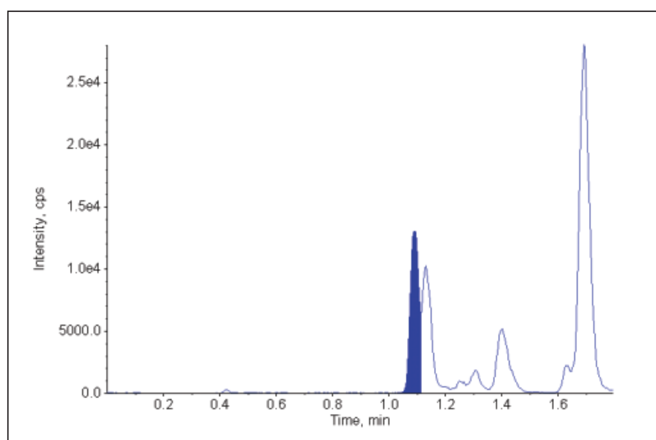


*Figure 2, SRM chromatogram for a Swedish plasma extract on a 50 x 2.1 mm, 1.7 µm ACQUITY BEH Shield RP18 column. The UDCA peak is shown in blue.*

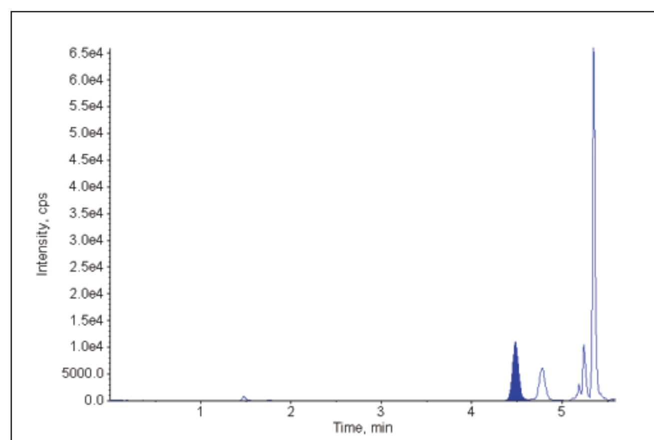


*Figure 3, SRM chromatogram for a Swedish plasma extract on a 150 x 2.1 mm, 1.7 µm ACQUITY BEH Shield RP18 column. The UDCA peak is shown in blue.*

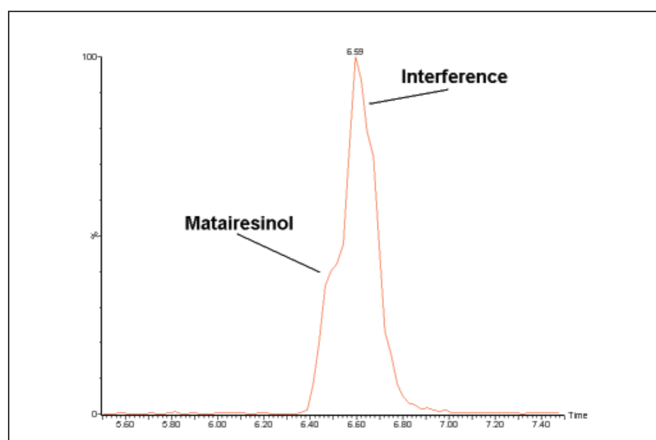


*Figure 4, SRM chromatogram for matairesinol in a urine extract using HPLC-MS/MS.*

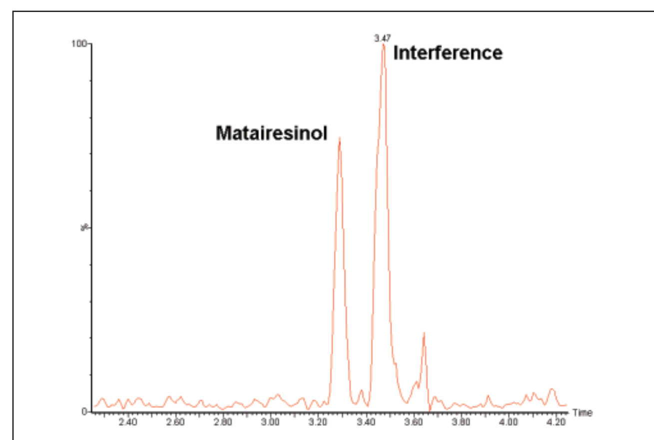


*Figure 5, SRM chromatogram for matairesinol in a urine extract using UHPLC-MS/MS.*

### UHPLC considerations

So what made this step change possible? Not just sub 2 µm silica particles but the combination of smaller particles with an ultra high pressure low-dispersion hardware system. Indeed, conventional HPLC systems probably only achieve 65% of the potential column efficiency. If you run the same column on a low dispersion system it may achieve up to 95% of the potential efficiency. However, it is questionable as to whether the manufacturers have yet mastered packing smaller particle columns as there is still significant variability in performance with columns of the same dimension and this is most apparent with short columns. In fact, in our experience these inconsistencies in packing tend to have less impact on the overall column performance in columns of 5 cm or longer when packed with 2 µm particles.

Column life-time is an important issue especially as these small particle columns are not cheap. There is clearly a greater potential for columns to block with the finer inlet frits. Aqueous buffers left at room temperature for more than 48 hours will start to grow bacteria which will begin to block the inlet frits resulting in a steady increase in column backpressure. However, by adopting the good HPLC house-keeping practices of 20 years ago i.e. filtering samples and buffers and regular replacement of mobile phases it is possible to get >2000 injections on a single column. However, fail to heed these good practices and column life-time is severely curtailed.

UHPLC method development strategies and procedures are very similar to those used for HPLC with in silico simulation/optimisation software available. Several of the column manufacturers provide application software that allows the transfer of conventional HPLC conditions to the equivalent UHPLC application. Method development is essentially no more difficult than that for HPLC and in fact – in most instances faster (< 5 days) using an orthogonal LC-MS column screening, followed by a temperature/gradient optimisation scheme. However the main area of caution is when comparing column selectivity. The selectivity of the sub 2 µm $C_{18}$ phase is very different to the equivalent 3 & 5 µm phases (e.g. Waters ACQUITY HSS T3 versus the Atlantis T3 columns). These differences are explained by the different particle geometries and the ability of column manufacturers to bond the stationary phase. Indeed, most exponents of UHPLC suggest that it is often easier to redevelop applications from scratch rather than try to transfer methods from HPLC.

So what about precision and accuracy of quantitation? Our experience, when directly comparing methods run by HPLC and UHPLC, is that the latter gives precision and accuracy at least as good as its more conventional counterpart. However, this does assume attention to detector optimisation. The reduction in peak width requires detector flow cells of smaller volumes or shorter dwell times when mass spectrometry is used.

### The future of UHPLC

So now UHPLC is firmly established, where is the technique going? There is clearly more to be had as improvements in the manufacturer's hardware and column robustness allow higher temperature and pressure separations, improving resolution and speed. More consistency in the packing of columns will result in greater assay reproducibility when using short columns. A greater range of bonded phases will become available through user demand. There will be greater pressure on manufacturers of conventional HPLC systems to optimise their systems. By reducing the dead-volume in pumps, dispersion in the system and selecting an appropriate small particle column, conventional HPLC systems can also deliver better resolution, speed and sensitivity gains. Temperature will be used much more routinely as a method development tool when high resolution is an important pre-requisite. Perhaps longer sub 2 µm columns operating at temperatures circa 90°C providing 'GC-like' separations will become less the realm of research and more routinely applied as an analytical solution.

However, one thing is for sure, the advent of sub 2 µm pressure resistant silica particles and high pressure-low dispersion HPLC pumps have brought chromatography sharply back into focus. Everywhere, analytical chemists are dusting off their chromatography books and reminding themselves what a Van Deemter plot looks like. They are reducing pump dead-volume, turning up the temperature on their column ovens and increasing mobile phase flow-rates.

### About HFL, the authors and acknowledgements

Established more than 40 years ago and located near Newmarket, HFL is the only laboratory in the world engaged in sports drug surveillance and contract research. HFL's clients therefore benefit from a unique perspective on drug development and surveillance coupled with an absolute focus on forensic quality science and commitment to excellent customer service. HFL provides laboratory services for all phases of drug development from discovery lead optimisation through to late-phase clinical trials and manufacture. Richard Houghton is a Principal Scientist at HFL focussed primarily on Pharmaceutical contract research whilst Philip Grace is a Principal Scientist working in the area of Nutrition and Sport.

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