Chromatography Solutions

Knowledge note #0011

Practical Ultra High Performance Liquid Chromatography (UHPLC)

INTRODUCTION

The potential to increase chromatographic efficiency and resolution along with significant savings in solvent cost and analysis time have driven the uptake of UHPLC to many application areas. This discussion outlines how instrument and column technologies continually evolve to meet the requirements of UHPLC, providing new options for chromatographers. Example data are provided to show the high speed and high resolution options of UHPLC. Advanced topics such as HPLC to UHPLC translations using free downloadable tools are also covered.

INSTRUMENT AND COLUMN TECHNOLOGY

The drive for increased chromatographic performance has led column manufacturers to continuously develop smaller and smaller particle sizes for liquid chromatography. Column efficiency is inversely proportional to the particle size, hence reducing the particle size increases the number of theoretical plates.
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Although smaller particles provide higher efficiencies, the trade-off is an increase in the operational back pressure. The introduction of commercially available high pressure capable instrumentation paved the way for the routine use of sub-2 micron particles. These small particles were shown to provide exceptional performance, particularly at flow rates higher than typically used with larger particle sizes, as demonstrated by their respective van Deemter plots (Figure 1, also see AKN0010: Band broadening and the van Deemter Equation). As shown, the optimum flow rate for 1.7 µm particles is greater than that of 3 and 5 µm particles. Additionally, 1.7 µm particles exhibit a flatter curve, meaning that they can still deliver high performance at higher flow rates. In contrast, larger particles show a sharper drop-off in performance when the flow rate is elevated beyond the optimum value.

The combination of the higher back pressure generated by smaller particles and the use of elevated flow rates necessitated the development of LC instrumentation capable of operating at ≥1,000 bar (~15,000 psi); a significant increase over the commonplace ~400 bar

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pressure limit offered by many HPLC instruments. The higher pressure requirements drove an extensive re-design of the pump and other system components to deliver stable liquid flow under such conditions. The entire flow path of UHPLC instruments has been re-engineered to ensure reliable operation at higher pressures. In addition, smaller format columns (e.g. 3.0 and 2.1 mm ID) are typically utilised to, firstly, increase the dissipation of frictional heat resulting from the use of high flow rates with small particle sizes, and secondly, to help achieve a reduction in solvent consumption.

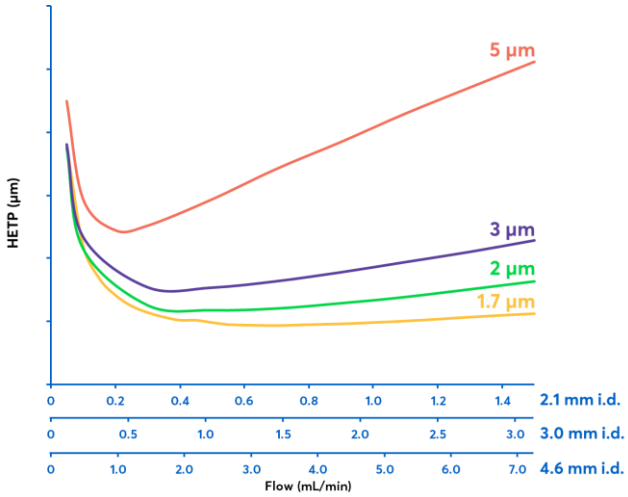

Figure 1: Van Deemter plots for various particle sizes. The minimum of each curve represents the optimal flow rate (i.e. highest efficiency) for that particle size.

The small peak volumes generated by these high efficiency, narrow-bore columns mean that UHPLC systems need substantially reduced extra-column dispersion compared to HPLC systems. Additionally, significantly improved optics / detector designs were needed, with lower volume cells and increased data capture rates, to be able to accurately describe analyte peaks and fully realise the practical gains in efficiency.

Column packing technology has also advanced to meet the demands of UHPLC. The particles packed into UHPLC columns must possess the mechanical stability to tolerate the very high pressures utilised in UHPLC. Packing techniques have adapted to ensure that high quality, reproducible and stable, packed beds can be reliably produced in UHPLC format columns.

ACE Excel UHPLC columns are packed using proprietary HSC™ (High Stability Column) packing technology to ensure that columns are robust and deliver excellent column lifetime under UHPLC conditions (Figure 2).

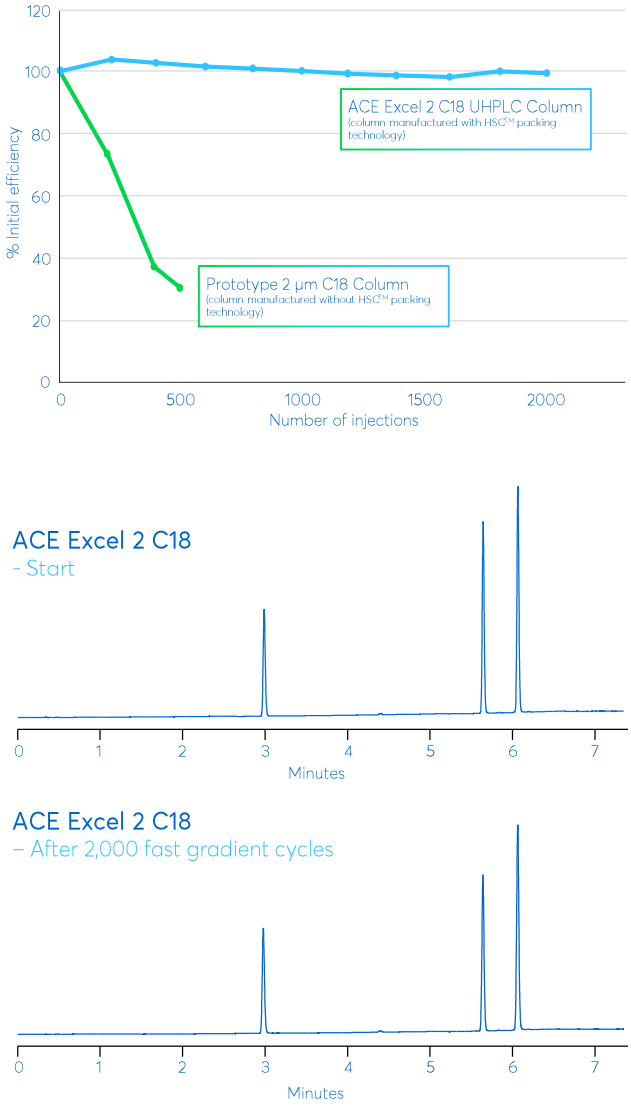

Figure 2: A 100 x 2.1 mm ACE Excel 2 C18 column, manufactured using HSC™ technology, was subjected to 2,000 cycles of a gradient with P_{max} of 1,000 bar (15,000 psi) and showed essentially no drop-off in efficiency, peak shape or retention. In contrast, a prototype column packed without HSC™ technology showed a rapid deterioration in performance.

SPEED OR RESOLUTION?

The use of exceptionally efficient sub 2-micron particles, alongside highly optimised UHPLC instruments capable of operating at high pressure, provides options to the modern chromatographer: improved speed and/or improved resolution (Figure 3). Short UHPLC columns can provide the same separating power as larger format HPLC columns but by using high flow rates (and therefore pressures) with the UHPLC column, separations can be achieved in a much faster timeframe (Figure 3a). The use of longer columns (and therefore higher pressure capabilities of UHPLC) enables the possibility to increase the resolution of the analytes in the separation (Figure 3b). Using this approach, very high column efficiencies and peak capacities can be achieved, that wouldn't be possible with HPLC. This approach is particularly attractive for complex samples with difficult to resolve peak pairs that require additional resolving power.

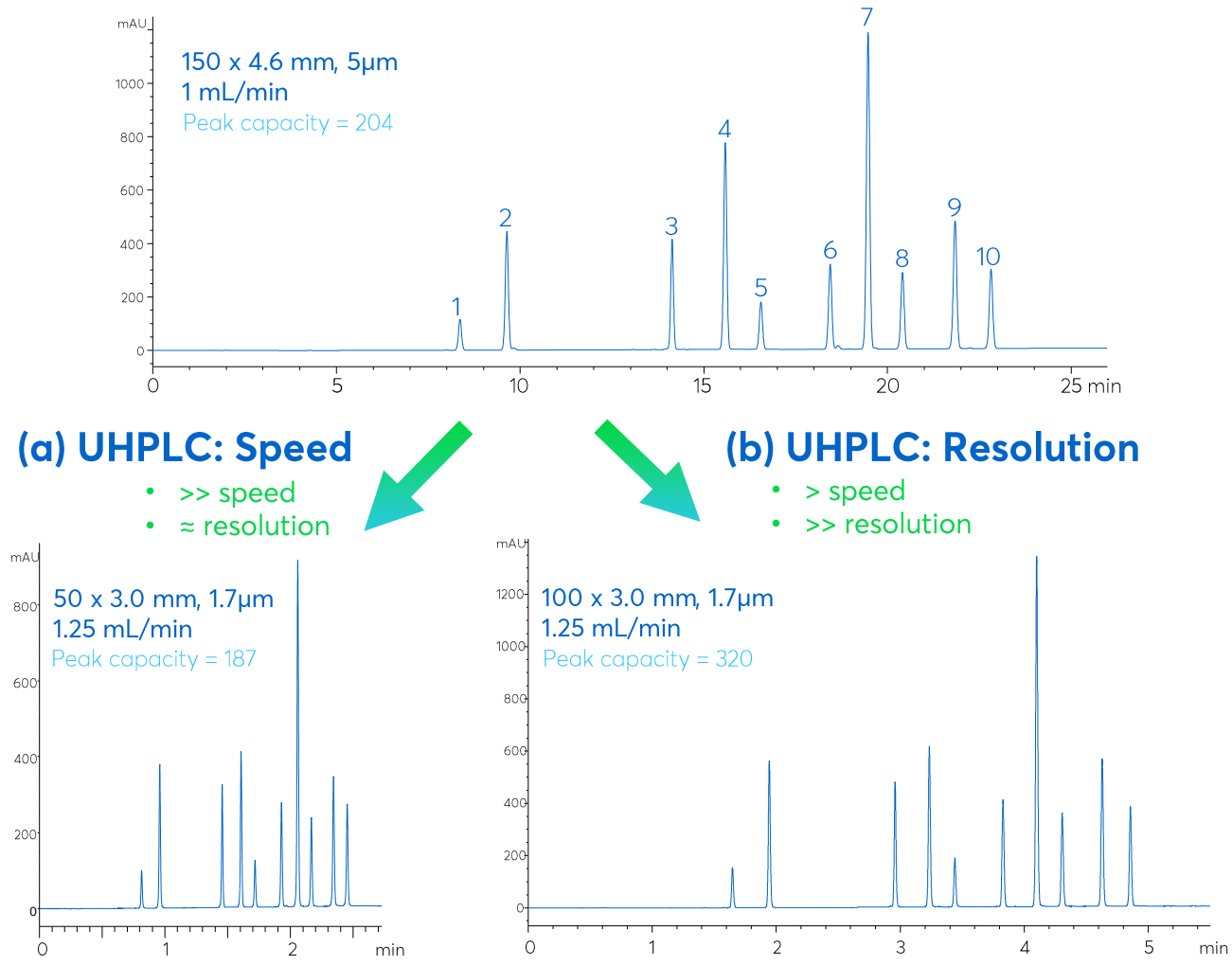

Figure 3: The use of UHPLC to achieve increased separation speed and/or increase analyte resolution. The top gradient separation of NSAIDs was performed by HPLC on an ACE Excel 5 SuperC18 column. Using UHPLC, the same separation can be achieved within 3 minutes using an ACE Excel 1.7 SuperC18 50 x 3.0 mm UHPLC column. Alternatively, the efficiency and resolution of the separation can be improved by running the gradient on an ACE Excel 1.7 SuperC18 100 x 3.0 mm UHPLC column. The method translations were achieved using the Avantor® ACE® LC Translator Tool.

HPLC TO UHPLC METHOD TRANSLATIONS

In many analytical laboratories, UHPLC is primarily used to generate chromatographic data faster for high-throughput, routine analyses and to accelerate method development. By using small format UHPLC columns packed with sub-2 micron particles and elevated flow rates, it is possible to perform highly efficient separations in a matter of minutes. Figure 4 shows the separation of six peptides using a 5 µm ACE C18 HPLC column. The separation required a 26-minute gradient profile, plus an additional 20 minutes post-gradient re-equilibration time, to give a total cycle time of 46 minutes. The same separation can be achieved by UHPLC on a 50 x 3.0 mm, 1.7 µm ACE UHPLC column using a 3.7-minute gradient profile and total cycle time of just 6.5 minutes. In this example, an 85% reduction in run time and solvent use is achieved through using UHPLC. Clearly, this increase in separation speed is highly attractive for increasing laboratory throughput and reducing solvent costs. Alternatively, many companies perform method development using UHPLC for speed and then translate the method back to HPLC for routine use.

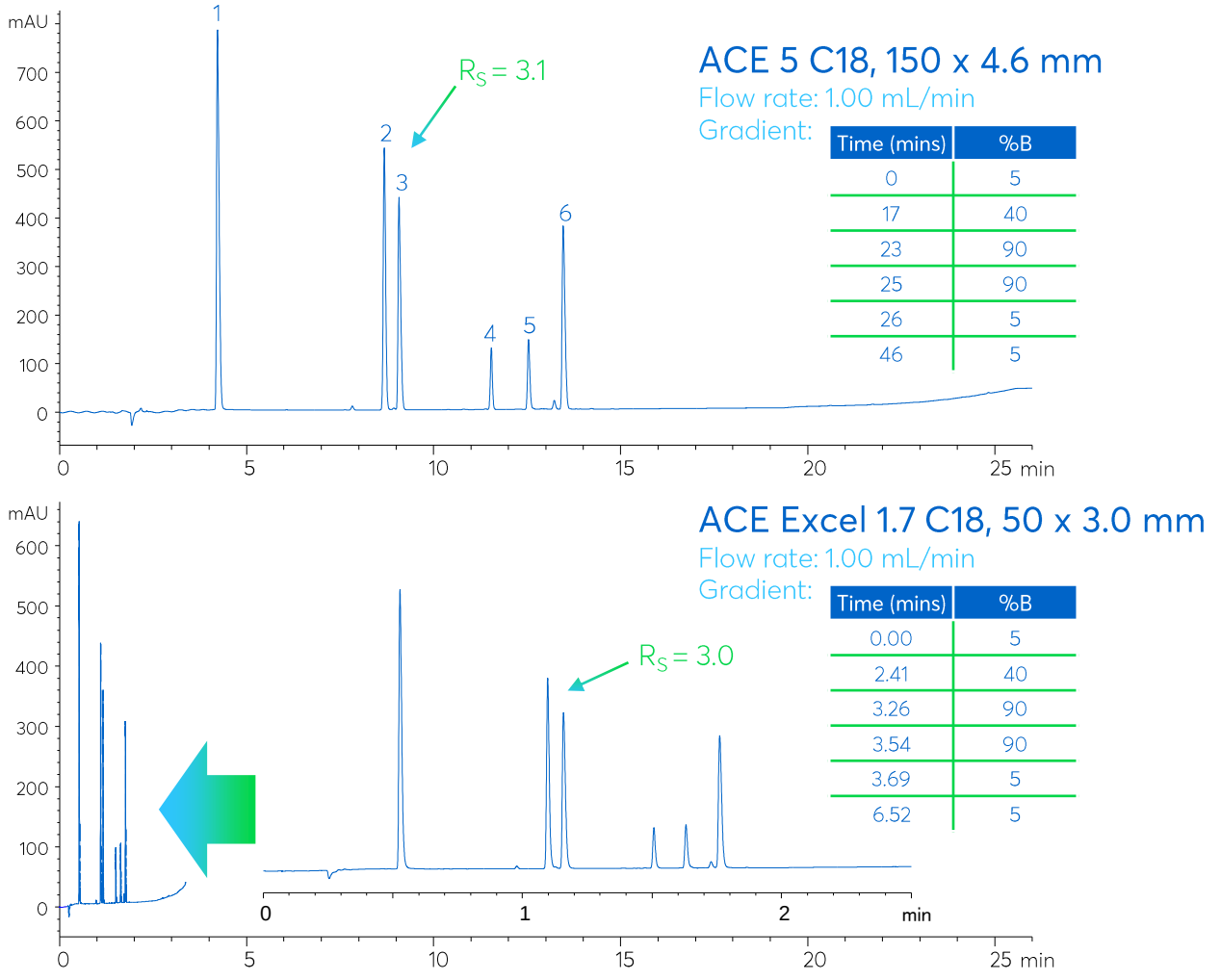

Figure 4, Top: separation of six peptides on an ACE 5 C18, HPLC column installed on an HPLC instrument.
Bottom: the same separation translated to an ACE Excel 1.7 C18, UHPLC column installed on a VWR Hitachi ChromasterUltra Rs UHPLC system. The method translation was achieved using the Avantor® ACE® LC Translator Tool. Mobile phase: A = 0.05% TFA in H_2O, B = 0.05% TFA in MeCN; Temperature: 60 °C; Detection: UV, 220 nm; Sample: 1. Gly-Tyr, 2. Tyr-Tyr-Tyr, 3. Val-Tyr-Val, 4. Oxytocin, 5. Angiotensin II, 6. Leu-enkephalin.

SIMPLIFIED METHOD TRANSLATION USING THE ACE LC TRANSLATOR TOOL

The translation of existing HPLC methods to UHPLC is now routinely performed and involves geometric scaling of the HPLC method to a UHPLC format column. The calculations for both isocratic and gradient method translations can be automatically performed using the free-to-download Avantor® ACE® LC Translator Tool. This MS Excel-based tool allows the user to input the HPLC column and method parameters, along with details of the UHPLC column that the method will be translated to. The UHPLC conditions are then automatically generated, from first principles formulae in the spreadsheet, without the need to manually perform the calculations. The new UHPLC conditions may then be inputted into the instrument and applied to the new column.

The ACE LC Translator Tool includes other useful chromatography calculators such as method transfer between instruments and for everyday, practical chromatography. For further details on method translation using the tool, please refer to AKN0023: Gradient Translation Using the ACE Translator Tool.

HIGH RESOLUTION SEPARATIONS

In addition to increasing the speed of analysis, UHPLC can also be utilised to achieve higher resolution by carrying out separations on longer columns packed with smaller particles. This approach can be used to resolve closely eluting analytes and is ideally suited to the analysis of complex samples and natural products. For example, a 100 mm column packed with 1.7 µm particles will offer twice the efficiency of a 50 mm 1.7 µm column. Taking this approach further and depending on the back pressure of the method, it is possible to couple multiple UHPLC columns to yield exceptionally high efficiencies. Figure 5 shows how this approach can be applied to improve resolution for the analysis of St. John's Wort - revealing additional sample detail (green boxes).

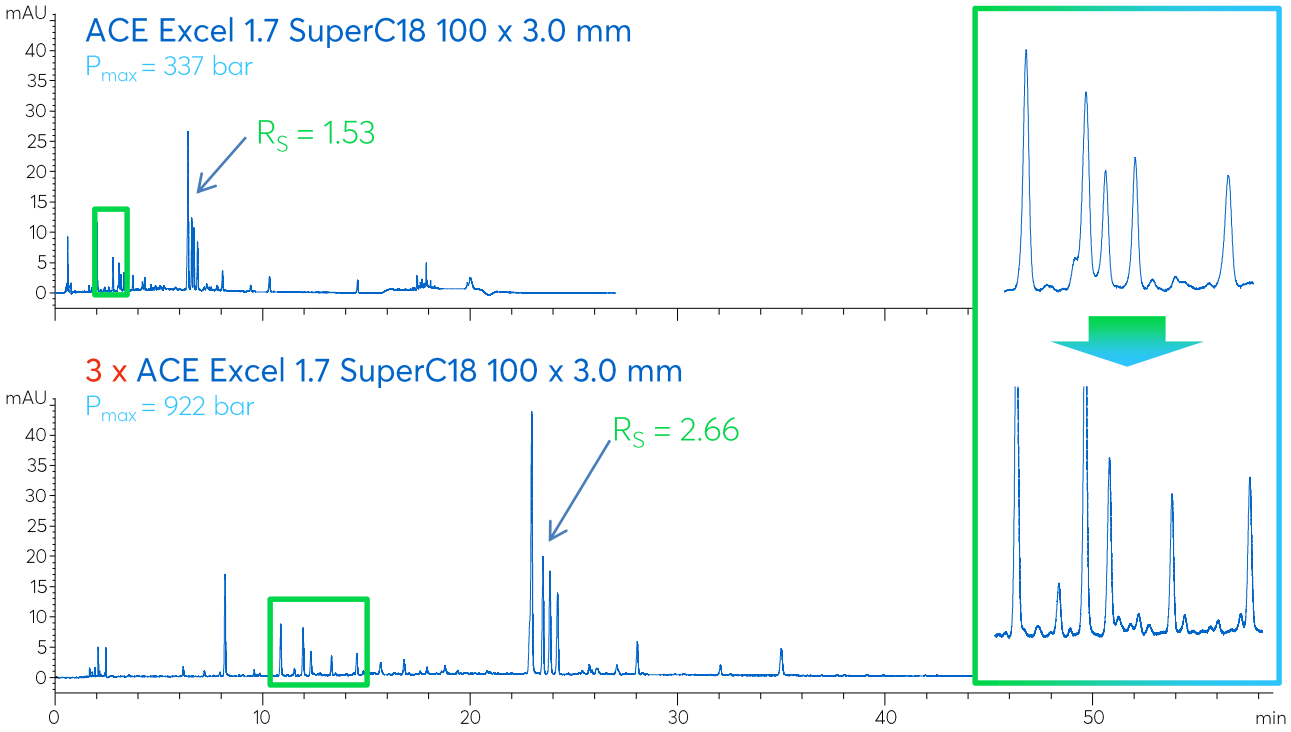

Figure 5, Top: analysis of St John's Wort on a 100 x 3.0 mm, ACE Excel 1.7 SuperC18 column with a 15-minute gradient time. **Bottom:** the separation was then run on three coupled ACE Excel 1.7 SuperC18 columns with a scaled gradient time of 45 minutes. This gives an effective column length of 300 mm. The inset shows the additional sample complexity revealed in the region of the chromatogram within the green boxes. Mobile phase: A = 0.1% formic acid in H_2O, B = 0.1% formic acid in MeCN; Gradient: 5-30 %B; Injection volume: 2 µL (100 x 3.0 mm), 6 µL (300 x 3.0 mm); Temperature: 60 °C; Detection: UV, 254 nm.

PRACTICAL CONSIDERATIONS IN UHPLC

To achieve the full benefits of sub-2 micron UHPLC columns, it is essential to appreciate that the column must be used with a UHPLC system that is optimised to reduce peak dispersion. The small peak volumes generated in UHPLC separations are extremely prone to extra-column band broadening effects caused by excessive system dispersion (usually a result of excessive dead volume being present within the system). Early eluting peaks are particularly prone to these dispersive effects. Low efficiency for early eluting analytes can therefore serve as an indicator that a system is not appropriately optimised. In situations where extra-column band broadening is suspected, switching to a wider bore column (e.g. changing from 2.1 to a 3.0 mm ID) can help to increase efficiency.

When installing a UHPLC column into an instrument, It is important to ensure that that good connections are made between the LC tubing and the receiving ports on the column to avoid introducing dead volume. Figure 6 shows the negative effect that incorrectly installing a column can have on peak symmetry and efficiency in UHPLC. It is recommended that re-useable fittings are used so that any differences in the port depth between different column brands are accounted for. For further information on how to correctly install UHPLC columns, please refer to AKN0006.

The narrow peaks produced in UHPLC separations require sufficiently fast data acquisition rates to provide enough data points to accurately describe the peaks. It is therefore important to pay close attention to detector settings in UHPLC to ensure that they are suitably optimised.

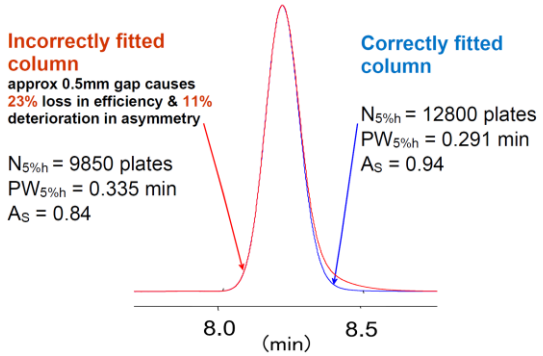

Figure 6: The effect of incorrect positioning of the inlet tubing, ferrule and nut at the column inlet.

UHPLC columns packed with small particles necessarily contain smaller frits than those used in HPLC columns. Additionally, UHPLC systems contain narrower ID tubing and may be equipped with very fine in-line filters. This means that UHPLC instruments and columns are more prone to becoming clogged by particulates. In order to reduce this risk, it is advisable to filter UHPLC mobile phases through 0.2 µm membrane filters to remove any particulates. Samples should also be suitably filtered prior to injection, as part of the sample preparation procedure. In-line filters can also help to protect the column from particulates from the sample and mobile phase and additionally, any that may originate from mechanical wear of system components. Table 1 provides details of ACE pre-column filters, which are compatible with any UHPLC columns.

Table 1: ACE UHPLC pre-column filters, 0.5 µm porosity - suitable for use up to 15,000 psi (1,000 bar).

Description	Part No.
Avantor® ACE® UHPLC pre-column filter	EXL-PCF05 (5/pk) EXL-PCF10 (10/PK)
Avantor® ACE® UHPLC pre-column filter, Waters® Acquity compatible	EXL-PCF05/ACQ (5/pk) EXL-PCF10/ACQ (10/pk)

CONCLUSION

Since it's inception, UHPLC has matured and is now an established and widely utilised technique in the analytical laboratory. The ability of UHPLC to accelerate both routine analyses and processes such as method development, along with the potential to enhance chromatographic efficiency and resolution has lead to the widespread adoption of the technique in a multitude of industry sectors. Although UHPLC requires a more in-depth knowledge of fundamental chromatographic concepts than traditional HPLC, an appreciation of the topics covered in this Knowledge Note will help to realise the potential of UHPLC.