Is Retention Time Precision Only Affected by the LC Pump?

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Overview

Purpose: Historically, the retention time precision of a liquid chromatography (LC) system was attributed to the pump of that instrument. With this poster we aim to illustrate the not only the pump is of importance but also the autosampler and detector technology.

Methods: We evaluated the retention time precision of an Thermo Scientific™ Vanquish™ UHPLC instrument. We evaluated the pump performance at demanding physical conditions, investigated the effect of a sample pre-compression and assessed the importance of sufficiently high data collection rates.

Results: Within this poster we clearly demonstrate that not only the pump performance influences the retention time reproducibility of multiple injections. Rather in modern UHPLC instrumentation the interplay between all modules, namely pump, autosampler, and detector, is of importance.

Introduction

Retention time precision is extremely important in being able to accurately compare results. Traditionally, retention time precision was always attributed to flow characteristics, such as consistency in flow rate and gradient precision, in an LC system. However, with UHPLC a point has now been reached where other factors, such as pressure and speed, play major roles. At injection the sample is pressurized from one bar to pressures above 1000 bar with the switch of a valve and peak widths can be so narrow that the data collection rate can become insufficient to accurately profile the peak. Finally, there are issues with gradient formation at pressures above a 1000 bar. In this work, we will look at all the aspects of a UHPLC system and discuss how chosen parameters can influence retention time.

We will show that more factors are important than just pump flow and gradient stability, and that it is possible to generate very accurate retention time precision at the top of the UHPLC operating range.

Materials

All experiments were performed with a Vanquish UHPLC system. Detailed methodological explanations are below of each of the respective chromatograms or in the figure caption.

Please not that certain instrument variables, such as Adaptive Thermal Effect Compensation (ATEC) or sample pre-compression can not be turned off by regular users of the instruments.

The Vanquish system consisted of:

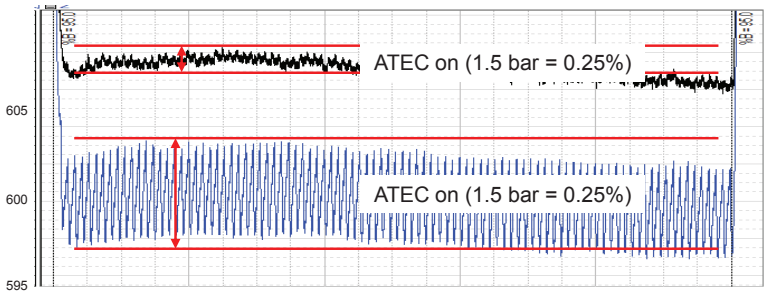
- Binary Pump H (P/N VH-P10-A)
- Split Sampler HT (P/N VH-A10-A)
- Column Compartment (P/N VH-C10-A)
- Diode Array Detector (P/N VH-D10-A)

The data was recorded using the Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software. Both versions Chromeleon CDS 6.8 and Chromeleon CDS 7.2 were used.

Pump Performance at 1500 Bar

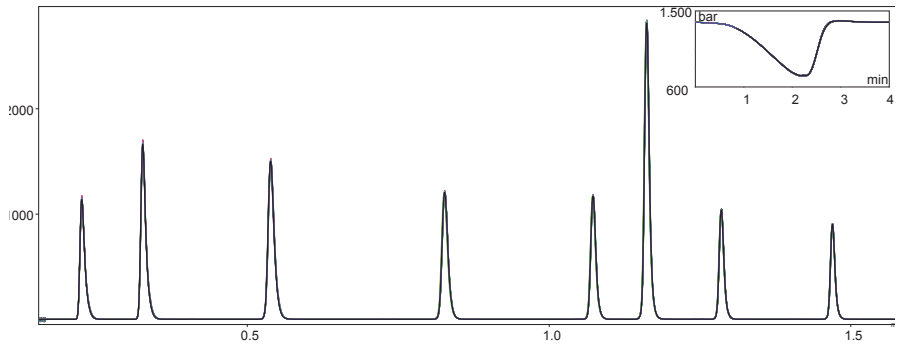
The main tasks of an LC pump is to deliver a constant eluent flow and to form gradients precisely. However, when delivering solvents under UHPLC conditions physical constraints need to be considered. A major effect is the production of additional heat during the compression of solvents which is e.g. more than 10 degrees when compressing methanol to 1000 bar. The cooling down procedure results in a volume contraction which would result in pump pressure pulsation. Obviously such pressure pulsation would influence the retention timereproducibility negatively. The effect can be reduced by employing an ATEC as shown in Figure 1.

FIGURE 1. Effect of compression heat on pump pulsation and the reduction of pump pulsation by applying the proprietary Adaptive Thermal Effect Compensation CH_3CN/H_2O 95/5 at flow of 2 mL/min.



Next to the flow consistency the gradient formation repeatability is crucial for highly reproducible chromatograms. That performance parameter can be easily evaluate in fast gradient separations.

FIGURE 2. Retention time reproducibility of a fast gradient separation. The insert displays the pressure trace of the chromatographic runs. Both plots are a overlay of 69 injections.



Column: Thermo Scientific™ Accucore™ Vanquish™ C18, 1.5 µm, 2.1 x 100 mm
Sample: Alkylphenone + Uracil
Eluent: A Water, B Acetonitrile
Gradient: 0—1.6 min 40-100% B, 1.6—1.9 min 100% B, 1.9—1.95 min 100—40% B, 1.95—4 min 40%B
Flow Rate: Up to 0.78 mL/min
Temperat..: 40 °C (still air)

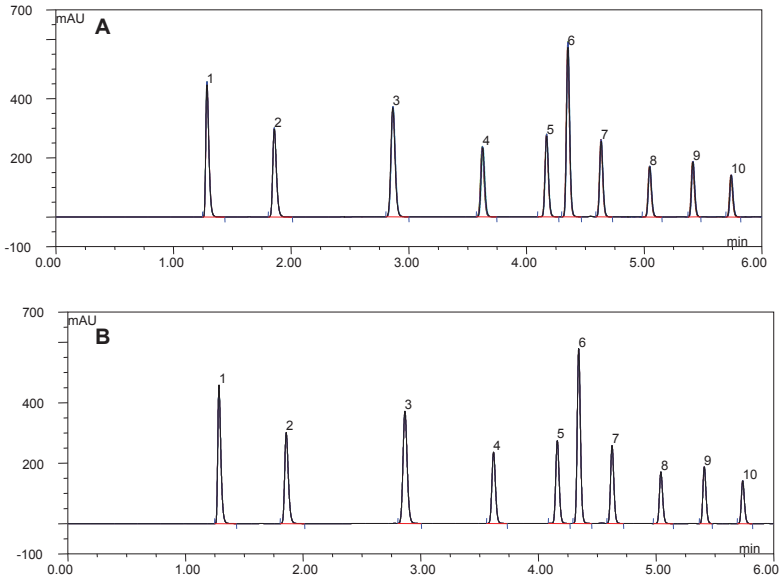
Figure 2 shows the separation of 7 phenones and uracil. The relative standard deviation of the retention time is as low as 0.17%, translating in 14 ms. This shows the excellent gradient formation at elevated pressures.

Sampler Influence on Retention Time Precision

The sampler of an LC instrument has to inject a liquid of ambient pressure into high a pressure flow path. Therefore, the sample is normally stored in the sample loop and injected to the column after a switch of the injection valve. During this process the sample is suddenly pressurized to system pressure and the system pressure drops due to the valve switching. This behavior creates flow inconsistencies and injection dispersion.

The Vanquish UHPLC system is capable to pre-pressurize the sample prior to the injection. This does not only positively influence the column lifetime but also does influence the retention time precision as shown in Figure 3.

FIGURE 3. Effect of sampe pre-compression on the retention time precisision. A without and B with pre-compression.



Column:	Thermo Scientific™ Hypersil GOLD™ C18, 1.9 µm, 2.1 x 100 mm
Sample:	Alkylphenone + Uracil
Eluent:	A Water, B Acetonitrile
Gradient:	0—0.4 min 40% B, 0.4—4.0 min 40—100% B, 4.0—4.8 min 100% B, 4.8—4.9 min 100—40% B, 4.9—10.0 min 40% B
Flow Rate:	0.26 mL/min
Temperat.:	25 °C (forced air)

This data clearly proves the positive influence of a sample pre-compression on retention time stability. In average the retention time precision improves by a factor of 6 when the sample pre-compression is enabled (Table 1).

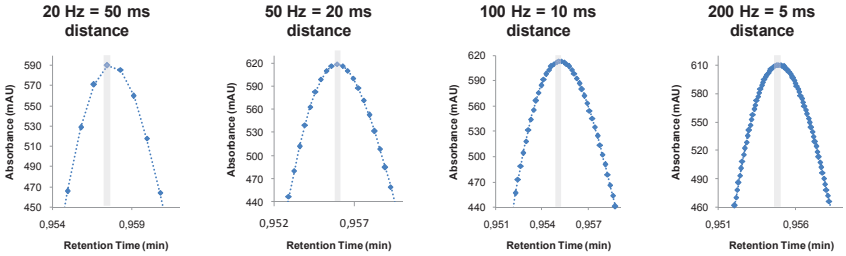
TABLE 1: Relative standard deviations (RSD) retention time of all substances from Figure 3. RSDs with and without sample pre-compression are directly compared.

RT RSD (%)	1	2	3	4	5	6	7	8	9	10
w/o pre-comp	0.040	0.026	0.050	0.096	0.035	0.033	0.033	0.039	0.044	0.040
w/ pre-comp	0.008	0.009	0.010	0.007	0.008	0.007	0.006	0.005	0.006	0.009

Role of Data Collection Rate

In guidelines for good chromatographic praxtice it is often described that a number of 30 data points is sufficient to characterize a peak adequately. Regarding peak area and peak shapethat is certainly the case. Regarding the peak RT, the retention time precision of the LC instruments needs to be considered. For example, a data collection of 20 Hz gives a signal every 50 ms. However, as shown earlier, the Thermo Scientific Vanquish instrument can deliver much lower retention time standard deviations. In case with retention time standard deviations around 20 ms, a data collection rate of 20 Hz would not be sufficient to take full advantage of the instruments potential.

FIGURE 4. Characterization of retention times at variable data collection rates. Please note that different retention times result from variable filter settings of the detector. The grey box represents a window of 20 ms, a retention time standard deviation easily obtain with the Vanquish system.

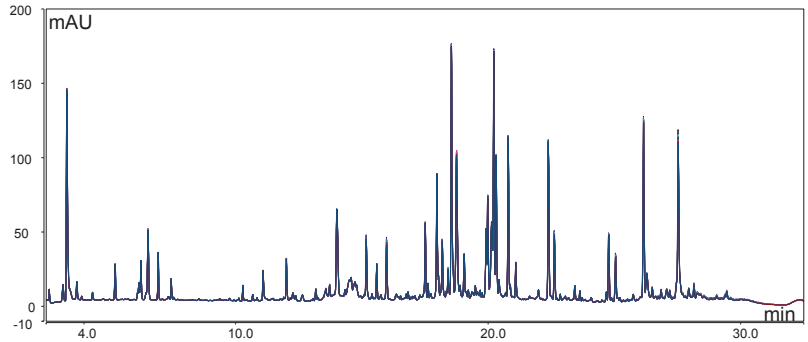


From Figure 4 it becomes obvious that 20 and 50 Hz are not sufficient to characterize the retention time of the peak in the same accuracy as the instrument is able to deliver it. For both cases, there is only one data point around the peak maximum. As the retention time will be very stable during multiple runs, the calculation of the retention time precision becomes statistically inadequate.

Influence on Complex Samples

Real life applications are most often much more complex than the examples given above. One of the most complex samples are digested proteins which are often used in biopharmaceutical industry for protein characterization in so called peptide maps. In this applications the peptide confirmation is often based on retention times, especially in process or quality control. As peak assignment is solely based on retention time, highest run-to-run retention time precision is required in order to avoid incorrect peptide identification, and drawing wrong conclusion. Thus, the analysis of complex samples can especially profit from the interplay of the Vanquish pump and autosampler.

FIGURE 5. Overlaid chromatogram of 13 repeated injection of a monoclonal antibody (mAb) tryptic digest.



Column:	Thermo Scientific™ Acclaim™ RSLC, 2.2 µm, 2.1 x 250 mm
Sample:	Tryptic digest of mAb
Eluent:	A: 0.05% TFA in Water, B: 0.04% in Acetonitrile
Gradient:	0—30 min 4%—50% B, 30 —31 min 50—90% B, 31—35 min 90% B, 35—36min 90—4% B, 36—45 min 4% B
Flow Rate:	0.4 mL/min
Temp.:	80 °C (forced air)

FIGURE 6. Retention time standard deviation measured for 13 repeated injections the mAb digest

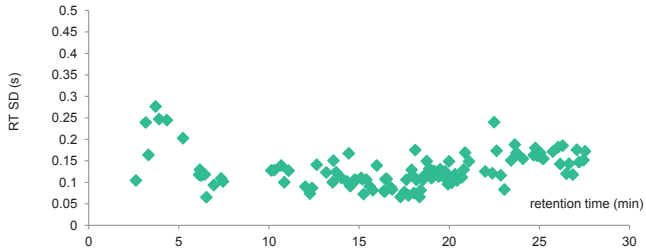
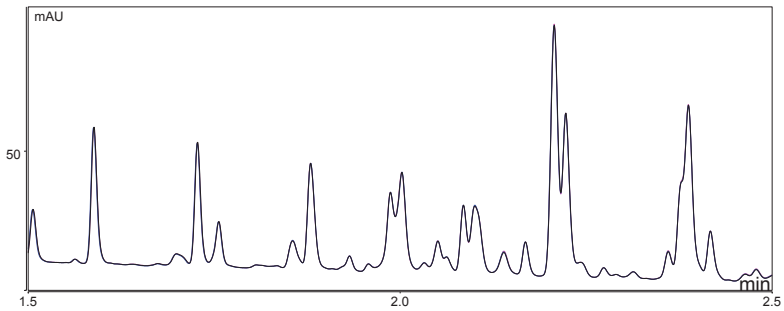


Figure 5 shows the highly repeatable injection of a digested mAb for a 30 min gradient time. For such complex samples the retention time stability is crucial for confident peptide identification. In that case the standard deviation ranged between 65 and 300 ms (Figure 6). The relative standard deviations were in over 30% of all peaks below 0.01%.

The Vanquish UHPLC system is able to highly reproducible perform chromatographic runs due to the sample pre-compression technology and very precise gradient formation. The gradient formation can be especially challenged by fast gradient during the analysis of complex samples. For the analysis of a mAb as shown in Figure 7 we applied a gradient time of 2.5 min only. For the overlay of three injections nearly no differentiation can be observed with the zoom into the chromatogram. Notable, the peak width at half maximum is on average in this application only 0.5 seconds.

Figure 7. Close view of 2.5 min peptide mapping of mAb digest (100 Hz). Overlay of 3 injections. Please note the excellent retention time and peak area/height reproducibility.



Conclusions

- The Vanquish pump compensates for physical effect at ultra high pressures. This results in a very constant flow delivery.
- The Vanquish Autosampler offers a sample pre-compression. That feature positively influences the flow consistency of the pump yielding higher retention time precisions.
- As retention time precision is significantly improved over other (U)HPLC systems with the Vanquish system, the data collection is becoming more important. Data collection rates as high as 100 Hz might be needed to fully display the systems reproducibility.
- The Vanquish UHPLC system delivers outstanding retention time reproducibility.

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