Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments

Mauro De Pra,[1] Ken Cook,[2] Mike Oliver,[3] and Carsten Paul[1]
[1]Thermo Fisher Scientific, Germering, Germany
[2]Thermo Fisher Scientific, Hemel Hempstead, United Kingdom
[3]Thermo Fisher Scientific, Runcorn, United Kingdom

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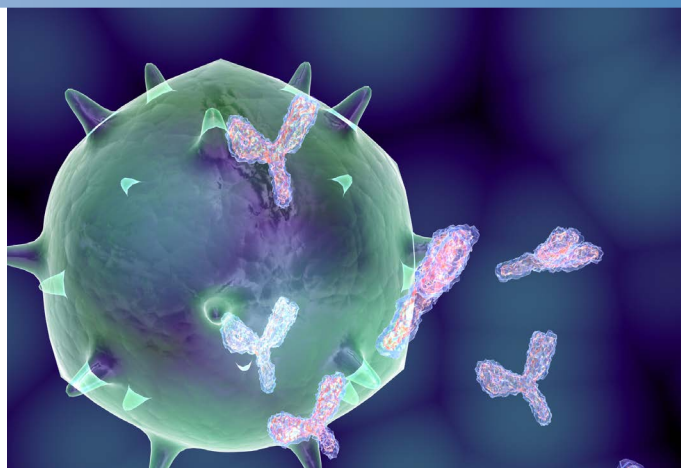
Key Words
Acclaim C18 RSLC, Biocompatible UHPLC, Biopharma, Biotherapeutics Characterization, Monoclonal Antibodies, Protein Digest, Vanquish UHPLC System

Goal

Provide an ultra-high retention time and peak area precision example of the separation of a mAb digest.



Introduction

Peptide mapping of digested proteins are of high importance when characterizing biotherapeutics. Peptide maps are utilized to confirm the expression of the intended amino acid sequence, to confirm genetic stability or to identify post-translational modifications, especially when interfaced with mass spectrometry. Reversed phase separation in combination with only UV detection is, however, still very common in stability studies, for in process measurements and quality assurance. In these cases peak areas, peak area ratio and retention times are sufficient to provide the required information. For highest confidence in the qualitative and quantitative results of such assays, the retention time as well as the peak area has to be extremely stable.

The Thermo Scientific™ Vanquish™ UHPLC system features a binary pump with extremely low pulsation ripple due to a brand new pump concept. In addition, the Vanquish UHPLC system pre-compresses the sample prior to the injection which results in a highly stable flow delivery. Thanks to these benefits, the Vanquish UHPLC system is capable of providing unmatched retention time precision. This retention time precision accompanied with a high peak area precision guarantees the analytical success for even challenging shallow gradient separations by a reliable peptide identification and quantification. In this work, the separation of peptides obtained from a therapeutic protein is provided. The retention time and peak area precision is evaluated for repeated injections.

Equipment

Vanquish UHPLC system consisting of:

- Binary Pump H (P/N VH-A10-A)
- System Base (P/N VH-S01-A)
- Mixer Kit, 200 µL, VH-P1 (6268.5120)
- Split Sampler HT (P/N VH-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Active pre-heater (6732.0110)
- Post column cooler, 1 µL (6732.0510)
- Diode Array Detector HL (P/N VH-D10-A)
- LightPipe™ flow cell, standard (10 mm; P/N 6083.0100)

Thermo Scientific™ Dionex™ Chromeleon™ Chromatogaphy Data System (CDS) software, version 7.2

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Protein Digestion

SMART Digest Kit (P/N 60109-101)

Experimental

Sample Preparation

1. Cetuximab® monoclonal antibody (5 mg /mL) was diluted 1:4 with the SMART Digest buffer to a final volume of 100 µL
2. The diluted sample was then added to a SMART Digest tube and left for 60 minutes at 70 °C
3. The digested sample was then centrifuged at 10,000g for 5 minutes and the supernatant was removed for chromatographic analysis

Conditions

Column:	Thermo Scientific™ Acclaim™ RSLC 120, C18, 2.2 µm Analytical (2.1 × 250 mm), P/N 074812
Mobile Phase:	A: 0.05% TFA in water, P/N TFA 85183 B: 0.04% TFA in 8/2 acetonitrile/water (v/v), P/N acetonitrile TS-51101
Gradient:	0–30 min: 4–50% B, 30-31 min: 50–90% B, 31–35 min: 90% B, 35–36 min: 90–4% B, 36–45 min: 4% B
Flow Rate:	0.4 mL/min
Maximal Pressure:	384 bar
Temperature:	80 °C; Forced Air Mode
Injection Volume:	5 µL
Detection:	214 nm Data Collection Rate: 20 Hz Response Time 0.2 sec
Flow Cell:	10 mm LightPipe™

Results and Discussion

The digestion was achieved utilizing the SMART digest kit. Using this approach the sample preparation time could be reduced significantly and total preparation time of the monoclonal antibody (mAb) digest was lower than 75 minutes.

The separation of the resulting peptides was obtained with a 30 minutes gradient, and a total analysis time of 45 minutes, including column wash with high organic eluent, and re-equilibration at initial conditions. Figure 1 shows the overlay of 13 consecutive injections of the same sample of mAb digest.

The results show excellent reproducibility across the whole chromatogram. On average, standard deviation (SD) was of the order of 0.13 seconds (0.00214 minutes). SD for some peaks was as low as 0.065 seconds; and did not exceed 0.3 seconds for any peptide.

The relative standard deviation was consistently extremely low (Figure 2). Out of 110 peaks automatically integrated by Chromeleon CDS, 34 had RSD smaller than 0.0100%, reaching the minimum value of 0.0060% for the peak at retention time 23.057 minutes. Please note that the early eluting peaks naturally have the highest retention time RSDs because of a mathematical disadvantage in the calculation.

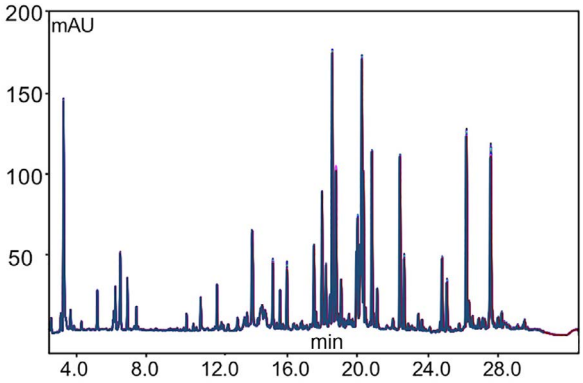

Figure 1. Overlaid chromatogram of 13 repeated injections of the mAb tryptic digest.

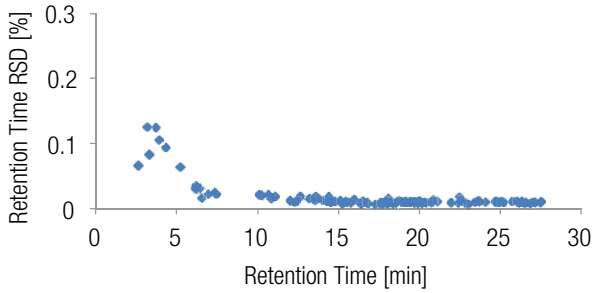

Figure 2. Retention Time RSD (%) relative standard deviation measured for 13 repeated injections of a mAb digest.

In addition, Table 1 gives the peak area reducibility as relative standard deviation.

The relative standard deviation of the peak areas was below 1.0% for all peptides. The average reproducibility was 0.4% highlighting the highly reliable sample injection and peak integration at challenging conditions.

Conclusion

Stability of retention time and peak areas is critical for a confident evaluation of chromatographic results and to avoid any misinterpretation. The Vanquish UHPLC system is extremely reproducible in both retention time and peak area reproducibility. The retention time precision provided by the system enables the analyst to deduce any change in retention time to an actual change of the sample structure. As shown, the peak area reproducibility provided by Vanquish will result in a maximal confidence of quantitative result. Consequently, the Vanquish system meets the requirements of demanding peptide mapping analysis.

Table 1. Peak area of six selected peaks eluting over the entire gradient and spanning a wide concentration range.

Retention Time (min)	Average Area (mAU*min)	RSD Area (%)
5.23	1.04	0.10
10.29	0.36	0.22
13.07	0.05	0.94
15.96	0.96	0.49
22.39	5.17	0.14
24.68	0.25	0.61

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