Technical Note 158

High Resolution Peptide Mapping for Biopharmaceutical Analysis

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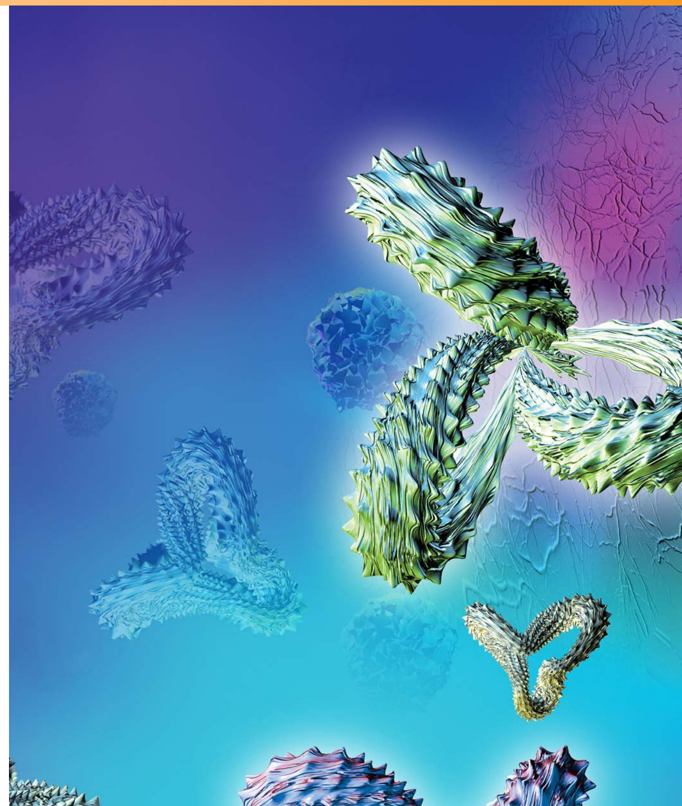
Keywords
Monoclonal Antibody, Protein Characterization, Biotherapeutics, Throughput, Peak Capacity

Goal
Provide guidelines to achieve high peak capacity for peptide separations without compromising throughput



Introduction

Reversed phase (RP) peptide separations are widely used in the biopharmaceutical industry. It is at present one of the most common analytical tools for the characterization of protein therapeutics, which are enzymatically digested. Peptide mapping provides confirmation of the correct amino acids sequence, and play a crucial role in the control of critical quality attributes. Furthermore, RP peptide separations are used in stability study or other fingerprinting based analysis, where the chromatogram of a tested protein digest is compared to a reference sample.

Proteins digests are often very complex samples, containing tens or even hundreds of peptides. Due to the sample complexity, gradient elution is mandatory. Peak capacity (nc) is often used as estimation of the resolving power of the method. nc can be interpreted as the theoretical maximum number of peaks that can be baseline resolved in the retention window. Long columns packed with small particles can be used to achieve large capacity values. Additionally, long gradient time, i.e. shallow gradient slopes can be used to achieve high peak capacity. This was extensively proven in bottom-up proteomics, where long gradients, up to 12 h long, have been used to fully exploit the resolving power potential of UHPLC capillary columns.[1] However, long gradient times have two main drawbacks. The first one is that throughput will dramatically decrease, a condition highly unfavorable for most of biopharmaceutical laboratories. The second drawback is that in-column peak dilution will decrease the detection sensitivity. Particularly when analysis is performed with UV detectors, the risk is that the low-level components fail being detected. It is therefore important to be able to achieve high peak capacity and at the same time keep the total run time reasonably short.

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Experimental

Instrumentation

- Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS UHPLC system equipped with:
  - DGP-3600RS pump (P/N 5040.0066) or LPG-3400RS pump (P/N 5040.0036)
  - VWD-3400RS detector (P/N 5074.0010)
  - Semi-micro flow cell for VWD-3000 Series, PEEK, 2.5 µL Volume, 7 mm Pathlength (P/N 6074.0300)
  - WPS-3000 TBRS well plate autosampler (P/N 5841.0020)
  - TCC-3000RS thermostatted column compartment Pre-column heater, 2 mL bio-compatible (P/N 5730.0000)

Chromatographic 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	
Mobile Phase B:	0.04% TFA in 8/2 acetonitrile/water	
Gradient:	Time (min)	%B
	0	4
	30	55
	31	90
	35	90
	36	4
	45	4
Flow Rate:	0.4 mL/min	
Temperature:	40, 60, 80 °C	
Injection Volume:	0.2–5 µL	
Detector:	UV 214 nm. Data Collection Rate: 10 Hz. Time Constant: 0.12 s	

Data Processing

Software Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software 7.2 or 6.8.

Peak Capacity Calculation

The peak capacity was calculated by using the formula:

$$\mathbf{n_c = \frac{t_w}{1.7 \cdot W_{1/2}} + 1}$$

where t_w is the selected retention window and $w_{½}$ is the peak width at half height. We calculated the peak capacity based on peaks of two protein digests; the digests were obtained from Bovine Serum Albumin (BSA) and a Monoclonal Antibody (mAb). Capacity calculation was based on 6 baseline resolved peaks selected across the retention window. Besides the criterion of baseline resolution, the peaks were chosen to cover the whole chromatogram as uniformly as possible, from low to high retention components. An additional capacity was calculated by using all peaks, including those not baseline resolved. Co-eluting peaks were included in the calculation; however, in case $w_{½}$ was larger than $w_{½}^{av} + 2\sigma$, where sigma is the standard deviation calculated for all peaks and $w_{½}^{av}$ is the average peak width at half height, the peak shape was checked. If peak shape distortion was clearly a result of co-elution then the peak was excluded from the calculation. The second approach is likely to produce an underestimation of the capacity, but serves as indicative control that the arbitrary choice of peaks did not yield to biased capacity evaluation.

All peak width used for the capacity calculation were average of multiple 6 injections, unless otherwise indicated.

Results and Discussion

Influence of Temperature on Peak Capacity

Peak capacity increases with the gradient time (Figure 1). Thus, investing time on the analysis pays off in terms of resolution. However, the capacity gain becomes progressively less important for longer gradients. Furthermore, pursuing very high peak capacity is certainly beneficial for very complex digests of multiple proteins, as found in proteomics samples for instance. However the separation of single proteins digests, which is a typical scenario in biotherapeutics analysis, is less demanding. Therefore, it makes sense to find a compromise between high resolution and analysis speed. Here we found that a 30 min long gradient provides a relatively fast analysis, and is capable of delivering peak capacity approaching values in the order of 400 (Figure 1).

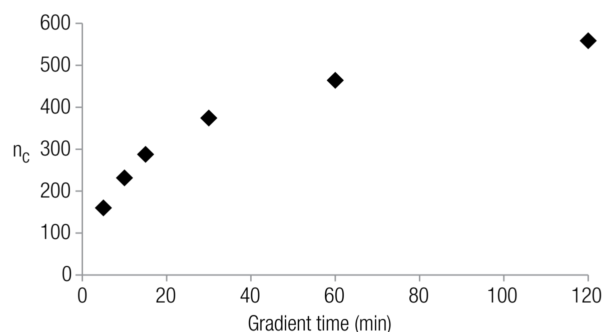

Figure 1. Peak capacity at different gradient time. Sample: BSA 5 mg/mL tryptic digest. Injection volume: 2 µL. Column temperature: 60 °C. Pump module: LPG-3400RS.

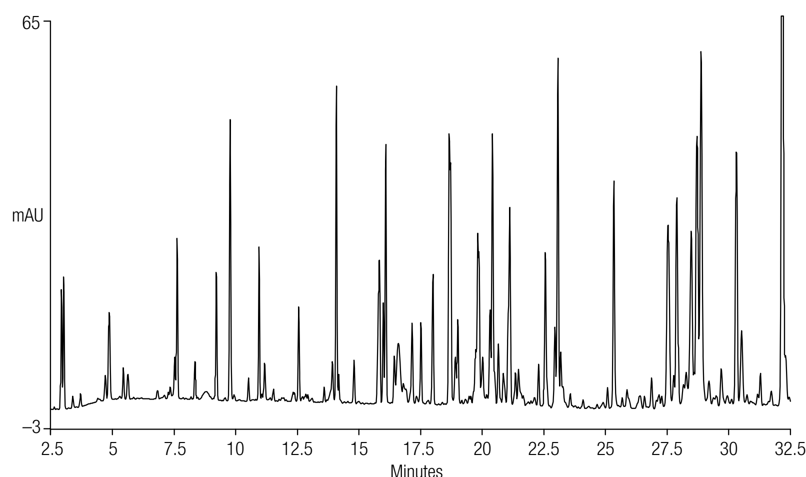

Figure 2. Separation of mAb 10mg/mL tryptic digest at 80 °C and 30 min gradient. Injection volume 1 µL. Pump module: DGP-3600RS.

Based on this preliminary observation, we decided to analyze a typical biopharmaceutical sample, such as a digest of a monoclonal antibody, with the same gradient length. It is well documented in the literature that temperature has major impact on the capacity[2,3] of peptides reversed phase. The mass transfer kinetic benefits from the increased diffusion rate of peptides at higher temperature, ultimately producing narrower peaks, thus higher capacity. Temperature increase from 40 °C to 60 °C lead to 12–18% increase in peak capacity (Table 1); heating to 80 °C increased the capacity by additional 6–9%. At 80 °C, the peak capacity was estimated to be 396. The large capacity is remarkable if considered the short gradient time of just 30 min, and total run time of 45 min, including column re-equilibration. This gradient length is therefore a good trade-off between analysis time and resolution, particularly if combined to thermostatting at 80 °C. Capacity values based on arbitrary chosen resolved peaks were higher than the values estimated with baseline resolved peaks, as expected. However, both estimation approaches provided consistent results, and did not affect data interpretation.

It is important to realize that when working at high temperature, proper mobile phase pre-heating is mandatory to avoid the detrimental effects on peak width of the thermal mismatch between the column and the mobile phase entering the column. The UltiMate 3000 BioRS system can be equipped with a bio-compatible solvent pre-column heater for operations at elevated temperature. The heater is installed inside the column compartment, and can be easily added or removed based on the requirements of the analytical method. The laboratory temperature was around 24 °C during the experiments; therefore the temperature difference between the solvent entering the column and the column oven was substantial. In the case of the peptide separation shown here, the thermal mismatch caused retention and diffusion rate variation inside the column that ultimately yielded to extra band broadening. The effect on capacity can be seen in Figure 3.

Table 1. Peak capacities achieved at different temperatures and 30 min gradient time. Sample: mAb digest 10 mg/mL. Injection volume: 0.2 µL. Pump module: DGP-3600RS. Capacity is calculated based on baseline resolved peaks, and by using all peaks according to the description in the text.

	Selected Peaks		All Peaks	
Temperature (°C)	**n_c**	**n_c increase Relative to lower column temperature (%)**	**n_c**	**n_c increase Relative to previous column temperature (%)**
40	314	—	309	—
60	372	18%	348	12%
80	396	6%	379	9%

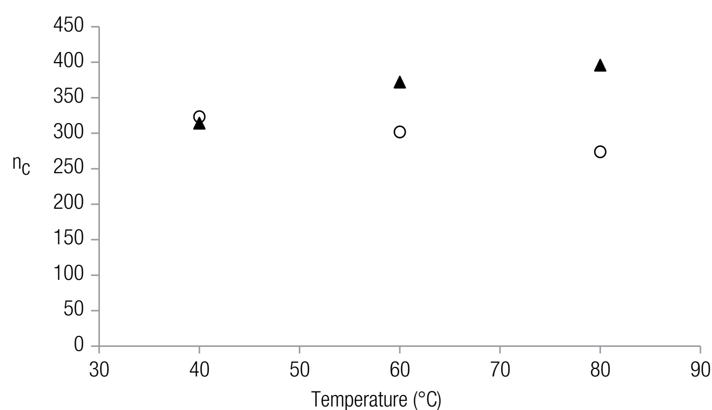

Figure 3. Estimated peak capacity at different column temperatures. Comparison between the use of pre-column heater (full triangles) and column thermostatting without pre-column heater (empty circles). Rest of conditions as in Table 1.

When working at 40 °C, the use of the pre-column heater did not influence final peak capacity significantly. However, at 60 and 80 °C, the thermal mismatch between the incoming eluent and the column caused considerable band dispersion. In fact, without pre-column heater, peak capacity decreased with temperature. A close look at the chromatograms recorded at different temperatures, with and without pre-column heater, reveals in details the effects of temperature and the importance of thermal balance between column and mobile phase. Changes in peak width and resolution can be observed in Figure 4 and Figure 5. For instance, peak-pair 1–2 was baseline resolved at 60 °C when the pre-column heater was used, but co-eluted when the solvent was not thermostatted. The loss of resolution was solely due to the extra peak dispersion induced by the thermal mismatch: for instance, the width at half height of peak 1 was 2.9 seconds with, and 3.2 seconds without the pre-column heater. Similarly, but even more evidently, at 80 °C peaks were narrower with the pre-column heater being used (Figure 5), and improved resolution could be observed throughout the chromatogram.

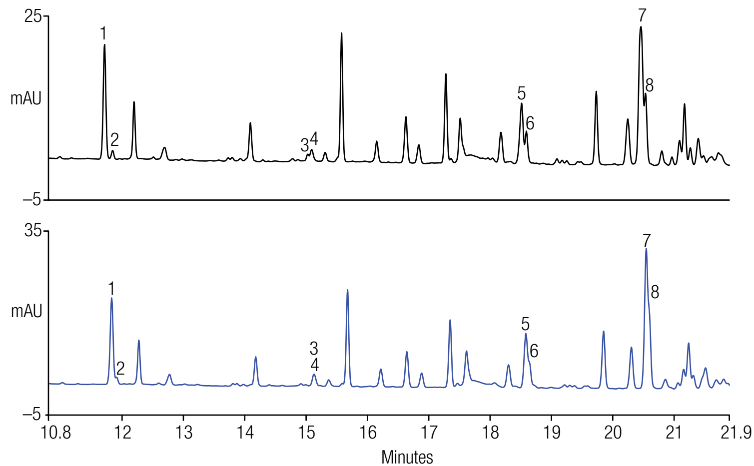

Figure 4. Chromatogram comparison at 60 °C with (top chromatogram) and without (bottom chromatogram) pre-column heater. Rest of conditions as in Table 1. The numbered peaks indicate instances where better resolution was achieved by using the pre-column heater.

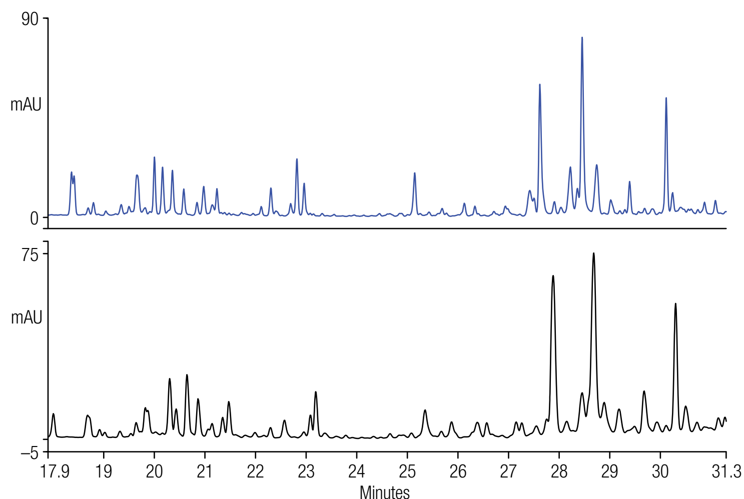

Figure 5. Chromatogram comparison at 80 °C with (top chromatogram), and without pre-column heater (bottom chromatogram). Rest of conditions as in Table 1.

Retention Time Precision

Retention time precision was evaluated for multiple injections of BSA digest and mAb digest. Retention time Relative Standard Deviation (RSD) of BSA peptides are visible in Table 2. Precision is excellent, and RSD values are always well below 0.1%.The peptide retention time precision of mAb peptides was highly reproducible as well. With RSD 0.076% or lower (Table 3). Measurements of Table 2 were collected with LPG-3400RS, whereas data of Table 3 were generated with DGP-3400RS. It can be concluded that both pumps provide highly reliable flow delivery.

Table 2. Retention time precision of BSA digest. Conditions specified in Figure 6.

Retention Time (min)	RSD% (n = 19)
6.486	0.095%
6.852	0.099%
8.078	0.058%
9.691	0.045%
12.099	0.037%
12.715	0.036%
13.481	0.029%
16.336	0.030%
18.065	0.030%
18.996	0.027%
19.251	0.021%
19.968	0.028%
22.288	0.026%
22.815	0.026%
24.917	0.022%

Table 3. Retention time precision of mAb Digest measured at 80 °C. Rest of conditions specified in Table 1.

Retention Time (min)	RSD% (n = 5)
7.369	0.076
9.473	0.074
13.838	0.036
15.895	0.056
18.370	0.042
20.000	0.057
22.964	0.028
25.150	0.034
28.459	0.049
30.112	0.022

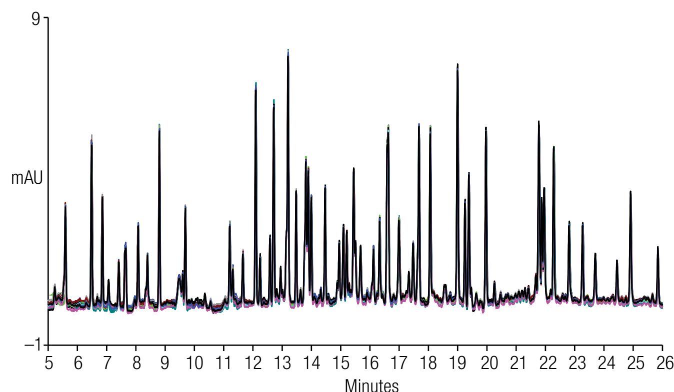

Figure 6. Overlay chromatogram of 19 repeated injections of BSA digest. Gradient time: 30 min. Rest of conditions as in Figure 1.

Conclusion

The UltiMate 3000 BioRS system in combination with the Acclaim RSLC C18 column provided excellent peak capacity for peptide separations. The combination of 30 min gradient time and column thermostatting at 80 °C, provided the resolution needed for digests of monoclonal antibody, and at the same time met the throughput requirements of biopharmaceutical laboratories. For analysis at high temperatures, the use of pre-column mobile phase heater is highly recommended. The excellent retention time is compatible with QC workflows, and any other fingerprinting-based peptide analysis.

References and Acknowledgements

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