Column Temperature Control in Peptide Mapping

Maximizing Peak Capacity by Keeping Your Column Hot the Right Way

Separation efficiency and peak capacity are important to analyze complex samples (e.g., peptide mapping). They depend on column plate number, system performance, and column temperature. In this article a new binary biocompatible UHPLC system is described that provides two column thermostatting modes, namely forced-air and still-air. For complex peptide mixtures of monoclonal antibody digests, still-air mode generates higher peak capacity compared to forced-air. Interestingly, the capacity gain is more evident at high temperature.

Reversed phase chromatography peptide mapping is extensively used for characterization and control of critical quality attributes of biotherapeutic proteins. In combination with MS, peptide mapping can provide the primary sequence of the protein of interest. Detection and quantitation of targeted peptides can be easily achieved by UV or MS detection. The high efficiency required to resolve the complexity of protein digests is provided by UHPLC columns. Long and shallow gradients can be applied to increase the number of resolved peptides. Although this is a well-known and proven approach to achieve higher peak capacity, unfortunately, long gradient times are hardly compatible with the throughput requirements of biopharmaceutical laboratories.

Other method tuning strategies are preferred to increase peptide peak capacity. An effective and recognized approach is to run the analysis at high temperature. The mass transfer kinetic of the relatively large peptide molecules is thereby improved, and better resolution achieved. Column compartment temperature can be controlled by two main approaches: forced-air or still-air mode, with the latter being the most common implementation on commercial UHPLC platforms. Forced-air thermostatting is preferred for method portability, which is in most cases, the transfer of a previously developed HPLC method to the UHPLC domain. Still-air is usually preferred to maximize the efficiency instead. With the new Thermo Scientific Vanquish system any of the two thermostatting modes are available in the same system thanks to the double functionality column compartment; the thermostatting mode can be set in the instrument method, based on the requirements and purpose of the analysis. Here we show how temperature, and the way of controlling it, affects the resolution of complex peptide mixtures, for a typical biopharmaceutical sample such as the tryptic digest of a monoclonal antibody.

Experimental

LC system: Vanquish UHPLC. Column: Thermo Scientific Acclaim RSLC 120 C18 column, 2.2 µm Analytical 2.1x250 mm. Mobile phase A: TFA 0.05% in water; mobile phase B: 0.04% TFA in 8/2 acetonitrile/water. Gradient program: from 4% to 55% B in 30 minutes. Injection volume: 1 µl. Sample: monoclonal IgG tryptic digest 2 mg/ml.

Peak capacity was calculated by the peak width at half height. All peaks were selected to calculate capacity even if co-eluting, unless the co-elution generated excessive peak distortion. Peak capacity was calculated by using the formula

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

It is well known from the literature that peak capacity in peptide mapping benefits from high temperature. The results shown here merely confirm this well-established knowledge. However it should be noticed that a peak capacity on the order of 400 was achieved at 80 °C, which is remarkable for a 30 minute gradient run (total run time 45 minutes, including column wash and re-equilibration).

The core-tool to obtain high resolution separation of complex mixtures is the column. The system is equally important, as it must generate the least possible extra column dispersion to ensure that the potential of the column is fully met. Among all the parts of a UHPLC that play a role on extra column band dispersion, the mo-

bile phase pre-heater is a critical component for separation at high temperature. The pre-heater must precisely adjust the temperature of the mobile phase prior to accessing the column. Any temperature mismatch can cause thermal inhomogeneity within the column that may be detrimental to efficiency. At the same time, the band dispersion generated by the additional volume of the pre-heater must be negligible. The low-dispersion active pre-heater installed in the UHPLC system allowed achieving high capacity in relatively short time.

Thermostatting Mode vs. Peak Capacity

The column thermostatting mode and its influence on peak capacity were evaluated at three temperature levels, namely 40, 60 and 80 °C (fig. 1). The forced-air mode allows fast column compartment temperature control, however when significant viscous heating is generated during the UHPLC run, this approach tends to cause a radial temperature gradient across the column, which causes retention and radial diffusion rate inhomogeneity. This produces additional band dispersion. The still-air mode approach instead, is clearly a less efficient heat-removal strategy, when significant viscous heating is generated. The slower viscous heating removal will create a temperature gradient with longitudinal orientation rather than radial, i.e. the column temperature will be lower at the inlet and higher at the outlet, but essentially constant for any given cross-section. With this approach the column intrinsic efficiency is better exploited. When the UHPLC method is transferred from HPLC, retention discrepancies are very likely to arise due to temperature difference between inlet and outlet.

The analysis of peak capacity at different temperatures with still-air and forced-air thermostatting, both showed the typical behavior, which is increase of peptide resolution with temperature. It also showed the expected efficiency increase that still-air mode thermal control allows in comparison to forced-air. However a closer look at the plot of figure 1 shows an interesting and also unexpected outcome: the efficiency benefits by still-air mode are increasingly higher with temperature. For instance, the capacity difference between still-air and forced-air is about 2.9% at 80 °C, 1.5% at 60 °C, but at 40 °C there is no actual capacity difference. This is unexpected as the most viscous-heating should be generated at 40 °C, where maximum back pressure was on the order of 650 bar. At 80 °C, where maximum pressure was on the order of 350 bar, the viscous heating effect was expected to be less important.

At 80 °C the average peak width at half height was about 2.64 seconds for still-air mode and 2.56 seconds for forced-air mode. The difference is not dramatic, but it was consistent throughout the retention window. The improvement may turn out to be useful in resolving critical pairs in the case of challenging separations. Detailed view of the chromatogram of Figure 2 shows several instances of better resolved peak pairs with still-air mode.

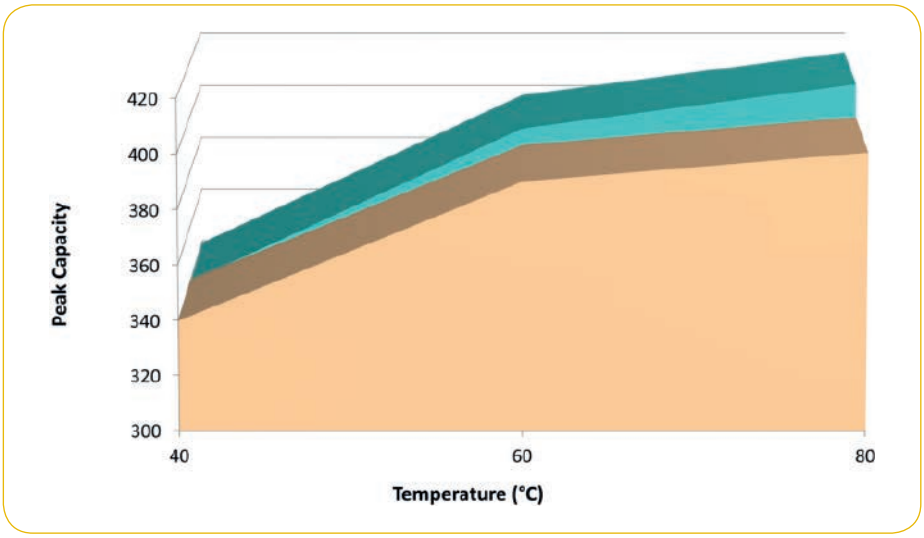

Fig. 1: Peak capacity obtained for gradient separation 30 minutes long. Still-air mode thermostatting (green) delivered higher peak capacity than forced-air mode thermostatting (tan) at 60 and 80 °C.

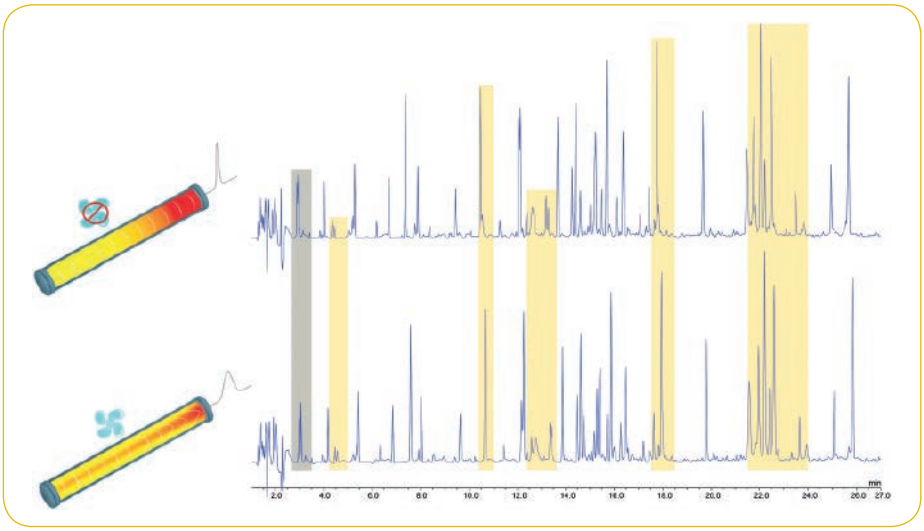

Fig. 2: Typical separation of a IgG digest at 80 °C with different thermostatting modes. The yellow boxes highlight the area in the chromatograms where the narrower peak width obtained with still-air thermostatting resulted in better resolution of closely eluting peaks. The grey area highlights where better resolution was found for forced-air thermostatting as the consequence of different selectivity, rather than peak-width improvement. On the left, the schematic view of the effect of viscous heating and temperature distribution in columns thermostatted by still-air (above) and forced-air (below). The color gradient from yellow to red indicates transition from low to high temperature.

Final considerations

Temperature control is crucial in peptide mapping. High temperature separations are preferred as peak capacity will increase thanks to the improved mass transfer kinetics. Another consequence of the temperature increase is that the retention of the vast majority of peptides will decrease. This effect will be beneficial to the elution of highly hydrophobic components, which would be otherwise detected as co-eluting wash-peaks with low-temperature methods.

A moderate but significant improvement of peak capacity was observed with still-air mode column compartment, particularly at high temperature. However forced-air mode still generated excellent results. The use of one or the other heating mode should be carefully considered in case of method transfer. For instance, when HPLC methods need to be converted to UHPLC (or the other way around), forced-air mode is the safest approach as it minimizes the chances of retention shifts due to viscous heating.

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