thermo scientific

TECHNICAL NOTE 72203

Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Authors

Martin Samonig, Sabrina Patzelt, Carsten Paul, Martin Rühl, and Remco Swart

Thermo Fisher Scientific, Germering, Germany

Keywords

Dual column, dual gradient, offline reconditioning, alternating column regeneration, Vanquish Tandem, Vanquish Flex, Vanquish Horizon, Q Exactive, monoclonal antibody, biotherapeutics, biosimilar

Goal

To demonstrate the use of the new Thermo Scientific™ Vanquish™ Tandem LC system and enable tandem analysis with two columns in parallel, addressing productivity and throughput improvement of existing LC-MS methods.

Introduction

Common liquid chromatography (LC) methods with gradient elution can be segmented into an analytical gradient section and a reconditioning section. The gradient section is responsible for the actual chromatographic separation, while the reconditioning section is used for the column wash and re-equilibration for the next injection (Figure 1). The process of column re-equilibration involves replacing the mobile phase between the particles (inter-particle), within the pores of the particles (intra-particle), and in the interfacial region between the mobile phase and stationary phase.[1] Good and accepted practice suggests using at least five column volumes to sufficiently equilibrate the analytical column.[2] If a column is required to be equilibrated with a buffered mobile phase or with a mobile phase containing an ion pair reagent, the required equilibration time is even longer. Depending on the column dimensions, gradient length, and flow rate, typically 10–60% of the total runtime is consumed by these column reconditioning steps within the gradient method.

ThermoFisher SCIENTIFIC

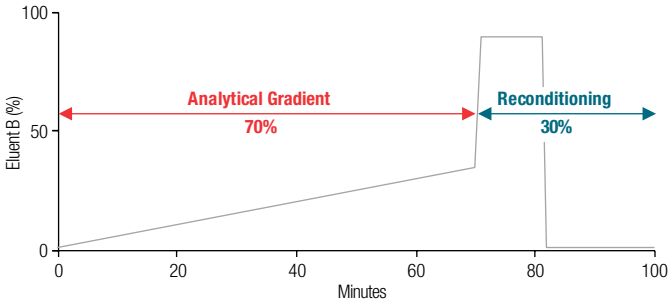

Figure 1. Gradient and reconditioning section of a common LC method.

Many UHPLC peptide mapping methods require lengthy periods of column washing and equilibration between separations. To possibly increase throughput and mitigate these delays without changing the chromatographic gradient section, a tandem LC approach with a two-pump setup and column switching capabilities can be implemented. In this setup one column is used for the ongoing separation, while the second column is switched offline from the mass spectrometer (MS) and simultaneously washed and conditioned for the next injection (Figure 2). The technique provides several benefits. First, throughput can be increased without changing existing (validated) methods. Second, with the latest instrument technology, a system suitable for the technique does not occupy any additional bench space (compared to a second LC-MS system). Third, laboratories can increase throughput without additional staff to operate multiple instruments.

Experimental

Consumables

- 2× Thermo Scientific™ Acclaim™ VANQUISH™ C18, column 2.1 × 250 mm, 2.2 µm, (P/N 074812-V)
- Fisher Scientific™ LC/MS grade water (P/N W/011217)
- Fisher Scientific LC/MS grade acetonitrile (P/N A/0638/17)
- Thermo Scientific™ Pierce™ LC/MS grade formic acid (P/N 28905)
- Thermo Scientific™ SMART Digest™ Kit (P/N 60109-101)

Sample pretreatment and sample preparation

A commercially available monoclonal antibody infliximab drug product (Hospira® UK Limited, Leamington Spa, United Kingdom) was supplied at a concentration of 10 mg/mL in a formulation buffer containing 0.05 mg/mL polysorbate 80, 50 mg/mL sucrose, 0.22 mg/mL monobasic sodium phosphate monohydrate, 0.61 mg/mL dibasic sodium phosphate dihydrate, and sterile water adjusted to pH 7.2 using sodium hydroxide or hydrochloric acid.

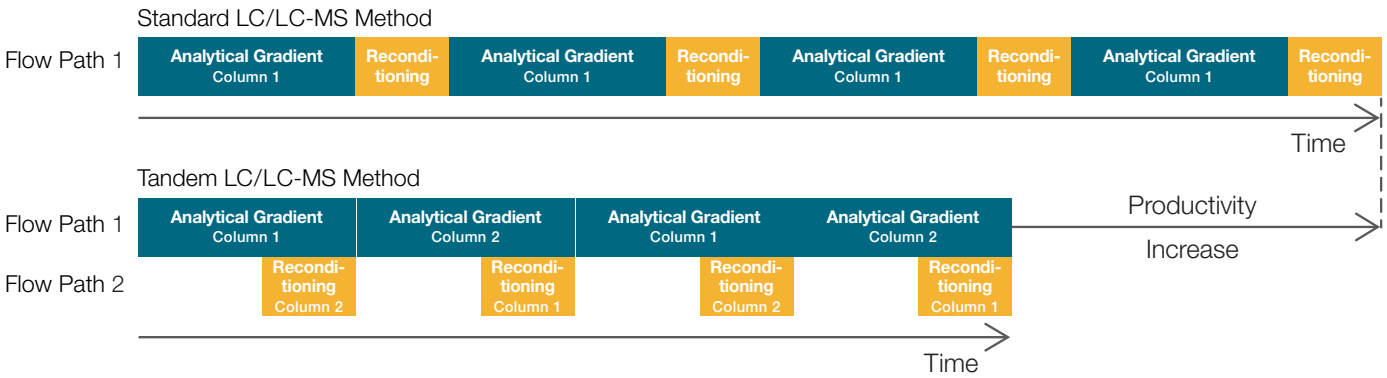

Figure 2. Standard LC-MS method compared to tandem LC-MS method.

SMART Digest Kit protocol

A 50 µL infliximab sample, adjusted to 2 mg/mL with water, was diluted 1:4 (v/v) with the SMART Digest buffer provided in the kit. The solution was then transferred to a reaction tube containing 15 µL of the SMART Digest resin slurry, corresponding to 14 µg of heat-stable immobilized trypsin. Tryptic digestion was allowed to proceed at 70 °C for 45 min at 1400 rpm. After the digestion, the reaction tube was centrifuged at 7000 rpm for 2 min, the supernatant was transferred to a new tube, and the centrifugation step was repeated.

The non-reduced sample was diluted with 0.1% formic acid (FA) in water to a final protein concentration of 100 ng/µL, and 1.0 µg was loaded on the column for all runs.

LC conditions

Instrumentation

Thermo Scientific™ Vanquish™ Horizon Tandem LC system consisting of the following:

- System Base Vanquish Horizon/Flex (P/N VF-S01-A-02)
- 2× Binary Pump H (P/N VH-P10-A)
- Split Sampler HT (P/N VH-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Variable Wavelength Detector F (P/N VF-D40-A)
- Flow Cell Semi-Micro, 2.5 µL, 7 mm light path (SST) (P/N 6077.0360)
- MS Connection Kit Vanquish (P/N 6720.0405)
- Vanquish Tandem LC Kit (P/N 6036.2020)

Figure 3 shows the Vanquish Tandem LC system chosen for this setup, consisting of two binary high pressure gradient pumps (HPG) used as an analytical pump and a reconditioning pump. The setup is configured for best chromatographic performance using the high-end Vanquish Binary Pump H, but is not limited to this particular pump type and can be also set up using other pump modules (e.g. Vanquish Binary Pump F (P/N VF-P10-A-01) or Vanquish Quaternary Pump F (P/N VF-P20-A)). All required capillaries and additional parts for this setup are defined in Table 1.

Separation conditions

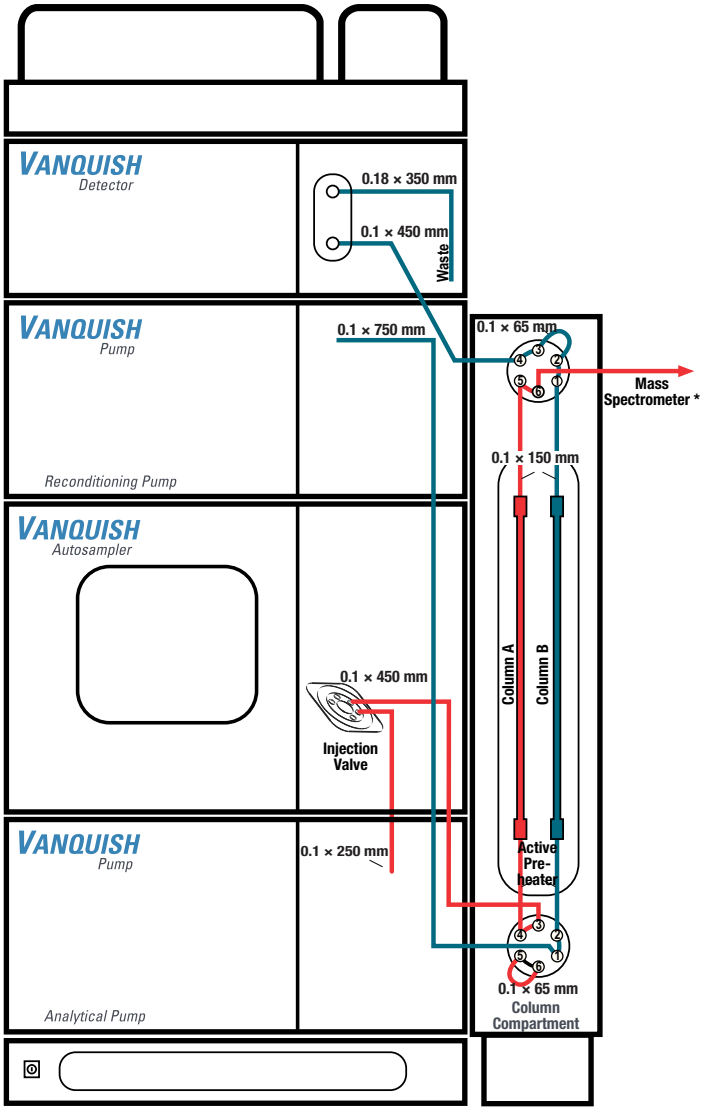

Figure 3. Vanquish Horizon Tandem LC system with 2-position/6-port (2p6p) valve configurations and required fluidic connections (for details see Table 1). * The recommended capillary to connect the LC to individual mass spectrometer depends on the setup and is defined in the Vanquish MS Connection Kit.

Table 1. Parts used for the Tandem LC-MS setup. Using a Vanquish VWD for reconditioning monitoring is a very specific use-case, so additional capillaries were needed for this setup. For all other Tandem LC or LC-MS configurations (independent from the pump type) the Vanquish Tandem LC Kit (P/N 6036.2020) contains all required parts and capillaries.

#	Amount	Product	PN
1	2	Biocompatible 2-position/6-port (2p6p) column switching valve	6036.1560
2	2	Viper Capillary, MP35N, biocompatible, 0.1 × 65 mm	6042.2306
3	2	Viper Capillary, MP35N, biocompatible, 0.1 × 150 mm	6042.2320
4	1*	Viper Capillary, MP35N, biocompatible, 0.1 × 250 mm	6042.2330
5	2	Viper Capillary, MP35N, biocompatible, 0.1 × 450 mm	6042.2350
6	1	Viper Capillary, MP35N, biocompatible, 0.1 × 750 mm	6042.2390
7	1	Viper Capillary, MP35N, biocompatible, 0.18 × 350 mm	6042.2337
8	2*	Active Pre-heater, 0.1 × 380 mm	6732.0110

* 1 already included in System Base Vanquish Ship Kits

Mobile phase A: Water + 0.1% formic acid
Mobile phase B: Water/acetonitrile (10:90 v/v) + 0.1% formic acid
Flow rate: See Table 2
Temperature: 60 °C, forced air
Detection: 214 nm
Gradient: See Table 2 and Figure 4

Table 2. LC gradient conditions for the separation of the mAb digest.

Analytical Pump			
Time [min]	A1 [%]	B1 [%]	Flow Rate [mL/min]
0.0	99	1	0.4
40.0	55	45	0.4
40.1	99	1	0.4
43.0	99	1	0.4
Reconditioning Pump			
Time [min]	A1 [%]	B1 [%]	Flow Rate [mL/min]
0.0	99	1	0.4
1.0	10	90	0.4
6.0	10	90	0.4
7.0	99	1	0.4
10.0	99	1	0.4
12.0	99	1	0.4
13.0	10	90	0.4
18.0	10	90	0.4
19.0	99	1	0.4
43.0	99	1	0.4

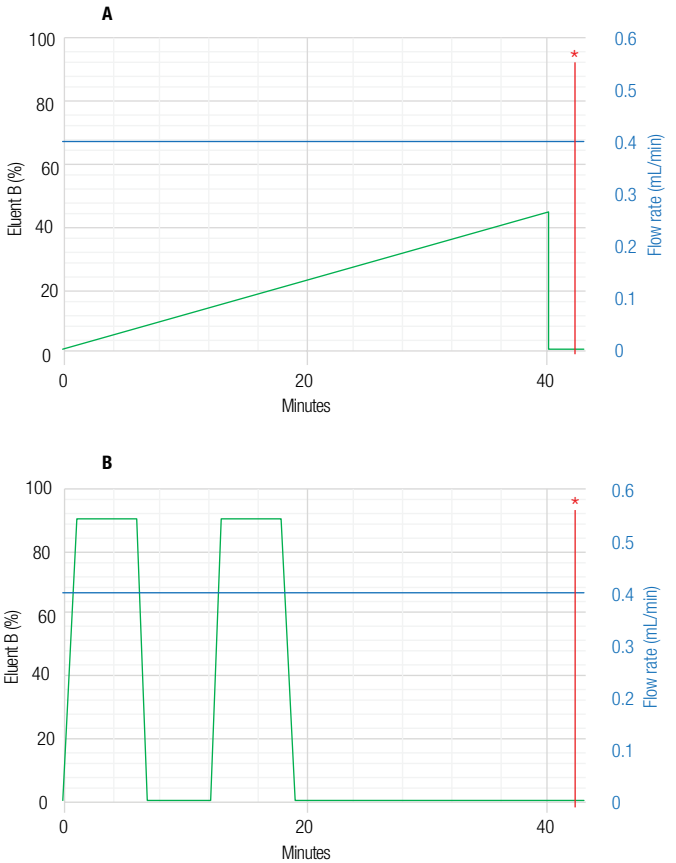

Figure 4.(A) Gradient Analytical Pump including the void volume purge at 40.0 min and (B) Gradient Reconditioning Pump including a multi-step wash section. The method was created using the Tandem LC method wizard implemented in Chromeleon. *Upper and lower valve switched position simultaneously at 40.9 min, with the "ColumnComp. NextColumn" command which is automatically inserted in the method from the Tandem LC method wizard.

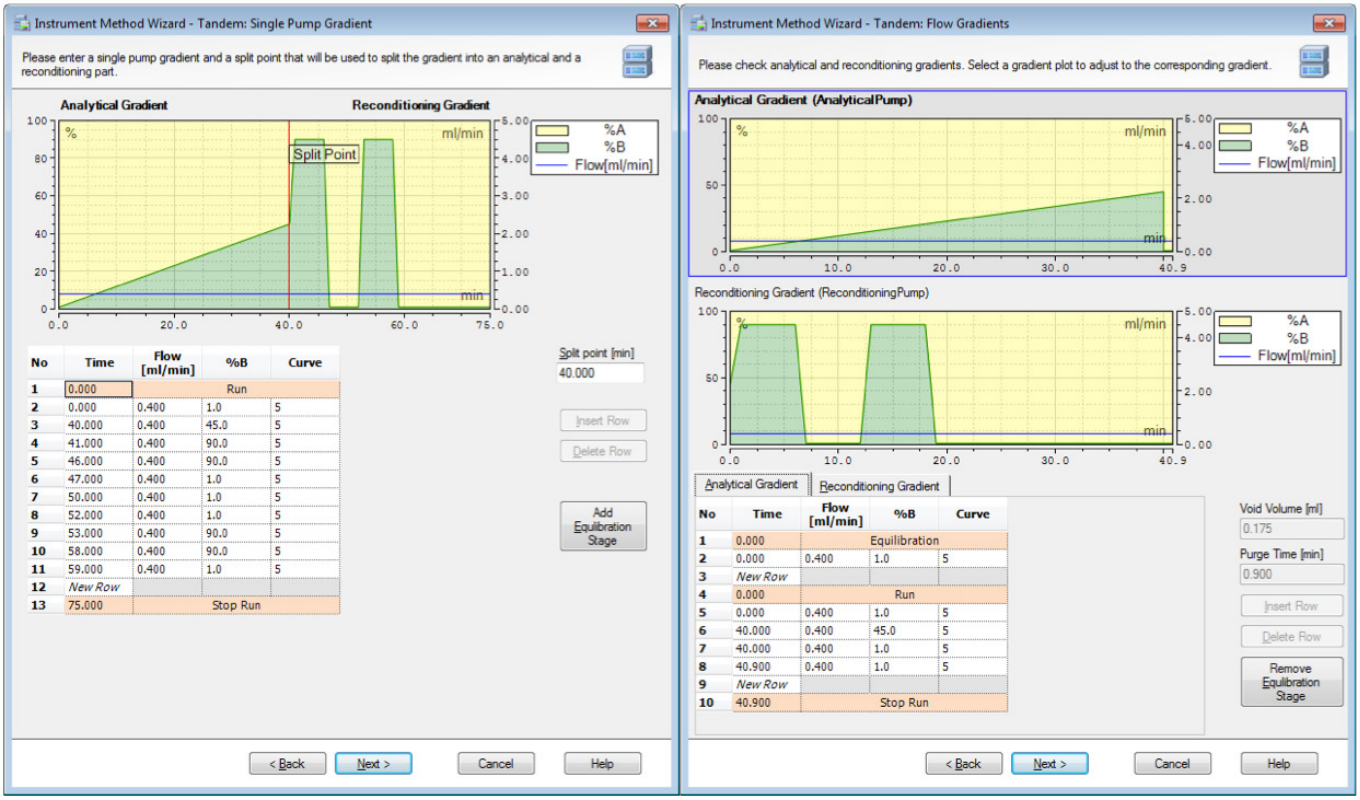

Figure 5. Chromeleon Tandem LC method wizard.

To enable the Tandem LC workflow, the corresponding fluidic description has to be selected within the Thermo Scientific™ Chromeleon™ Chromatography Data System software. Implemented in Chromeleon is also a specific Tandem LC method wizard (Figure 5), which enables straight forward transformation of existing methods into Tandem LC methods. The lower and upper switching valve of the Thermo Scientific™ Vanquish™ Thermostatted Column Compartment (VTCC) was used to switch between the two flow paths and two analytical columns (Figure 6). The analytical pump was utilized to deliver a water/acetonitrile +0.1% formic acid gradient (Table 2) to separate the peptides on one column. Simultaneously, the second column, offline from the mass spectrometer, was subject to a multi-step wash and equilibration gradient delivered by the reconditioning pump (Table 2) prior to being switched online for the next injection. A multi-step wash section with repeated up and down gradients was used to increase the washing efficiency and to reduce carryover for very big and non-polar tryptic peptides.[3] At the end of the gradient, the analytical pump was set to initial conditions at 40.0 min to perform a void volume purge and equilibrate the fluidics from the analytical pump to the lower switching valve for the next injection. At 40.9 min, the lower and upper switching valve changed the position and the next sample was immediately injected on the pre-equilibrated analytical column. In LC-MS setups a UV detector is not always needed. This setup used the Thermo Scientific™ Vanquish™ Variable Wavelength Detector (VWD) to monitor the reconditioning step to ensure that no peptides were eluting from the column during this stage, and to confirm proper column equilibration.

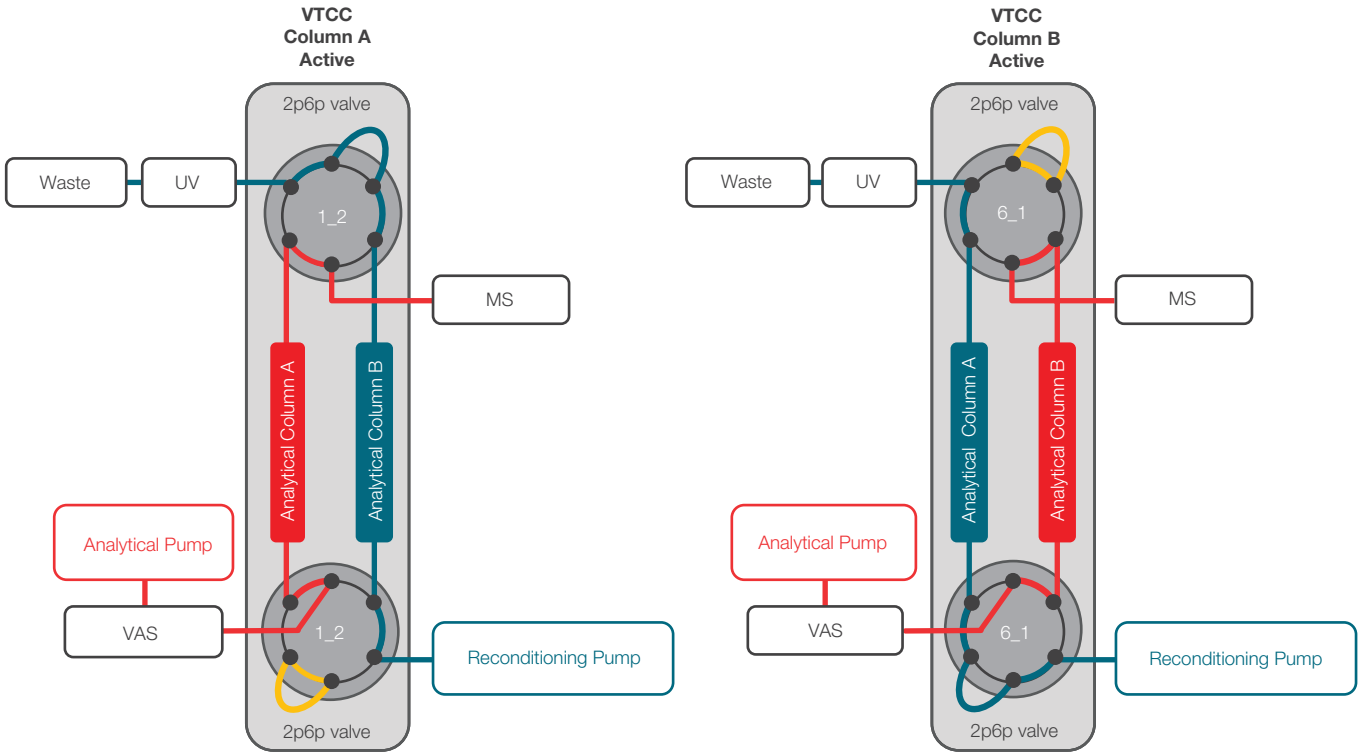

Figure 6. Flow schematic for tandem operation with two flow paths. One for analysis (red) and one for off-line column wash and re-equilibration (blue).

MS conditions

The Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap mass spectrometer was used for detection. The detailed MS source and method parameters are given in Table 3.

Data processing

The data were acquired and analyzed with the Thermo Scientific™ Chromeleon™ Chromatography Data System, version 7.2.8.

Results and discussion

Using the Vanquish Horizon Tandem LC system peptide mapping experiments, or more precisely for the separation of the tryptic digested monoclonal antibody infliximab, gave reproducible and confident results as demonstrated in the total ion current (TIC) chromatogram overlay of five replicates (Figure 7) on two analytical columns with automated alternating column regeneration.

Retention time relative standard deviation (RSD) values

Table 3. MS source and method parameters.

MS Source Parameters	Setting
Source	Ion Max source with HESI-II probe
Sheath gas pressure	45 psi
Auxiliary gas flow	12 arbitrary units
Vaporizer temperature	350 °C
Capillary temperature	350 °C
S-lens RF voltage	60 V
Source voltage	3.5 kV
MS Method Parameters	**Setting**
Method type	Full MS only
Full MS mass range	140–2000 *m/z*
Resolution settings	15.000 (FWHM at *m/z* 200)
Target value	3e6
Max injection time	200 ms
Microscans	1
SID	0 eV

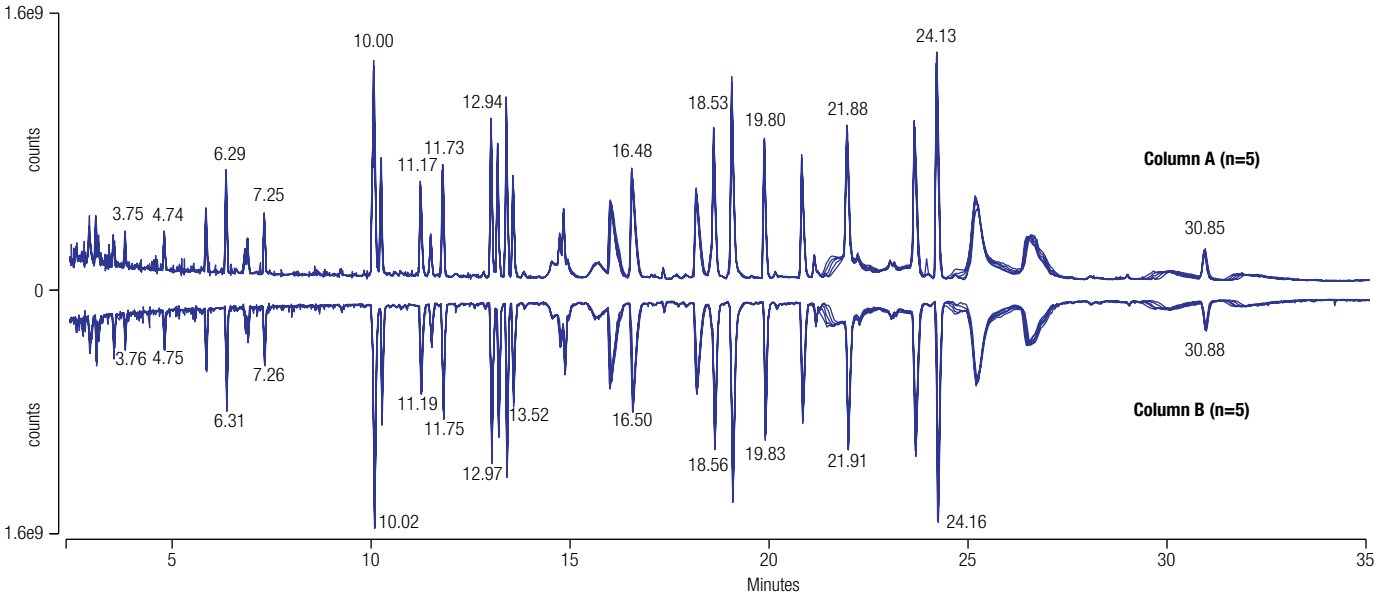

Figure 7. Reproducible results for the Vanquish Horizon Tandem LC - Q Exactive HF setup, showing the overlay of five TIC chromatograms for the separation of digested infliximab using the SMART Digest Kit.

below 0.11% were achieved for the UHPLC system in tandem column operation compared to 0.045% and 0.039% for the single column setup. Polar tryptic peptides eluting between 0 and 14 min had the highest RSD values up to 0.18%, and the heavy chain peptide (D151-Y183) at 30.85 min had the lowest with 0.064% (Table 4). The average absolute retention time shift between column A and column B was 0.023 min (relative, 0.18%) and shows that peak assignment based on retention time is not impaired. An average peak area RSD value of 2.47% demonstrates the suitability for quantitative data analysis using the tandem LC setup.

The advanced wash and reconditioning method used

Table 4. Reproducible results for the Vanquish tandem LC - Q Exactive HF setup with detailed RSD values for infliximab tryptic peptides for column A/B in tandem and single column operation based on the TIC chromatograms shown in Figure 7.

Column A			Column B					Column A/B	
RT [min] n=5	RT RSD [%] n=5	Area RSD [%] n=5	RT [min] n=5	RT RSD [%] n=5	Area RSD [%] n=5	Abs. RT Shift Column A to B [min]	Rel. RT Shift Column A to B [%]	RT RSD [%] n=10	Area RSD [%] n=10
3.75	0.18	2.72	3.76	0.13	1.64	0.005	0.13	0.16	2.12
4.74	0.054	3.53	4.75	0.11	5.05	0.010	0.21	0.14	4.24
6.29	0.072	2.33	6.31	0.037	1.14	0.020	0.32	0.18	2.19
7.25	0.018	4.94	7.26	0.033	4.72	0.016	0.23	0.12	4.94
10.00	0.032	3.05	10.02	0.037	1.75	0.023	0.23	0.12	2.35
11.17	0.040	3.96	11.19	0.047	2.70	0.022	0.20	0.11	3.22
11.73	0.043	1.64	11.75	0.007	2.59	0.025	0.21	0.12	2.15
12.94	0.014	4.19	12.97	0.012	1.61	0.023	0.18	0.10	3.03
13.49	0.028	1.66	13.52	0.025	3.11	0.024	0.18	0.10	2.36
16.48	0.056	1.02	16.50	0.031	0.78	0.024	0.14	0.087	0.91
18.53	0.019	1.94	18.56	0.020	1.90	0.027	0.15	0.080	2.35
19.80	0.019	0.50	19.83	0.016	0.78	0.029	0.15	0.078	0.62
21.88	0.028	4.35	21.91	0.0075	1.71	0.033	0.15	0.083	3.78
24.13	0.025	1.52	24.16	0.030	0.60	0.031	0.13	0.072	1.09
30.85	0.039	1.56	30.88	0.039	2.00	0.031	0.10	0.064	1.74
Average	**0.045**	**2.59**		**0.039**	**2.14**	**0.023**	**0.18**	**0.11**	**2.47**

in this study enables significant reduction of protein/peptide column carryover and can also be individually optimized by reducing or increasing the flow rate during the method. The UV trace used to exclusively monitor the wash and equilibration step of the reconditioning pump showed reproducible results for all runs (Figure 8).

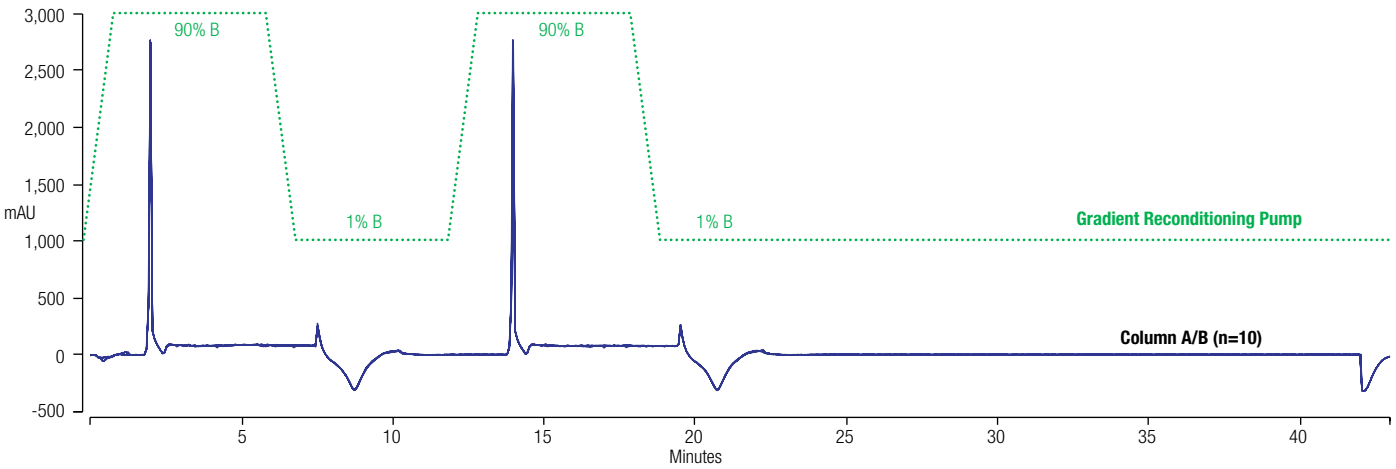

Figure 8. Overlay of ten chromatograms of the tandem LC reconditioning step.

Conclusions

The Vanquish Horizon Tandem LC system enables a throughput increase up to 40% without changing the actual gradient of the existing peptide mapping method. The retention time RSD values are below 0.11% for the tandem and single column operation. In this study, peptide mapping methods were used to demonstrate the capabilities of a tandem LC setup, but it can be applied to other methods and samples as well. Both Chromeleon version 7.2.8 and Thermo Scientific™ SII for Xcalibur™ version 1.4 support the Vanquish Tandem LC workflow.

References

1. Fletcher, M. R., Foley, J. P., Kinetic Model of HPLC Column Re-Equilibration after Gradient Elution. Conference Paper, Eastern Analytical Symposium 2015, Somerset, NJ.
2. Van Middlesworth, B. J., Dorsey, J. G., Re-equilibration Time of Superficially Porous Silica Based Columns in Gradient Elution Reversed Phase Liquid Chromatography. *Journal of Chromatography A,* 2011, *1218*(40), 7158–65. doi:10.1016/j.chroma.2011.08.030.
3. Freiser, H.H., Nowlan, M.P., Schmuck, M.N., The Use of Stabilized Silica Support with a Short Alkyl Chain for Reversed-Phase Preparative Chromatography of Proteins, Micra Scientific, product bulletin number 10, 1988.

Find out more at **thermofisher.com/Vanquish**

TN72203-EN 0124M