Tandem UHPLC Operation for High-throughput LC-MS Peptide Mapping Analyses

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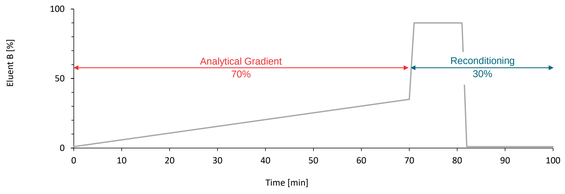
ABSTRACT

To demonstrate the use of the newly developed Thermo Scientific™ Vanquish™ Horizon Duo UHPLC system for Tandem LC-MS workflows and enable peptide mapping analysis with two columns in parallel, addressing productivity and throughput improvement of existing LC-MS methods.

INTRODUCTION

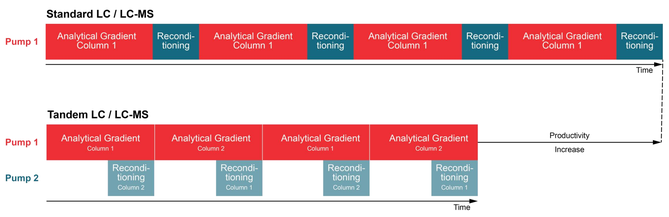
Common liquid chromatography (LC) methods with gradient elution can be segmented into an analytical gradient section and a reconditioning section. The gradient section is responsible for the actual chromatographic separation, while the reconditioning section is used for the column wash and re-equilibration for the next injection (Figure 1). The process of column re-equilibration involves replacing the mobile phase between the particles (interparticle), 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.

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.

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



MATERIALS AND METHODS

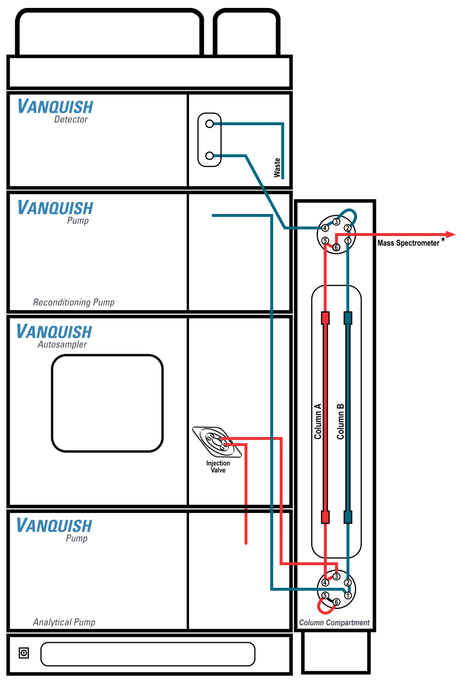
Sample Preparation

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. A 50 µL infliximab sample, adjusted to 2 mg/mL with water, was diluted 1:4 (v/v) with the Digest buffer provided in the Thermo Scientific™ SMART Digest™ 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.

Test Method(s)

The Vanquish Horizon Duo UHPLC system for Tandem LC-MS workflows with two 2.1 x 250 mm i.d. Thermo Scientific™ Acclaim™ Vanquish™ C18 columns and gradients of water and ACN with 0.1% formic acid each were used to separate the peptide mixtures. Figure 3 shows the Vanquish Duo UHPLC system chosen for this setup, consisting of two binary high pressure gradient pumps (HPG) used as an analytical pump and a reconditioning pump.

Figure 3. Vanquish Horizon Duo for Tandem LC-MS workflows.



To enable the Tandem LC workflow, the corresponding fluidic description has to be selected within the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS). Implemented in Chromeleon CDS is also a specific Tandem LC method wizard (Figure 4), 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 3). The analytical pump was utilized to deliver a water/acetonitrile + 0.1% formic acid gradient (Table 1) 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 1) 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 (VVWD) 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 4. Chromeleon CDS Tandem LC method wizard.

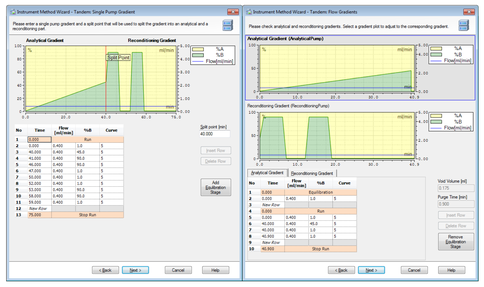


Table 1. LC gradient conditions for the separation of the mAb digest.

Analytical Pump			
Time [min]	A1 [%]	B1 [%]	Flow Rate [mL/min]
0	99	1	0.4
40.0	55	45	0.4
40.1	99	1	0.4
40.9	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
40.9	99	1	0.4

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 2.

Table 2. MS source and method parameters.

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

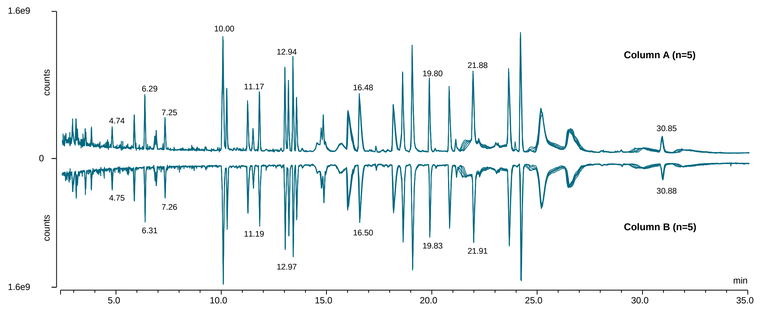
Data Analysis

The data were acquired and analyzed exclusively with the Chromeleon CDS, version 7.2.8.

RESULTS

Using the Vanquish Duo Tandem LC-MS workflow for 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 5) on two analytical columns with automated alternating column regeneration.

Figure 5. Reproducible results for the Vanquish Duo system for Tandem LC - Q Exactive HF MS setup, showing the overlay of five TIC chromatograms for the separation of digested infliximab using the SMART Digest Kit.



Retention time relative standard deviation (RSD) values 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 3).

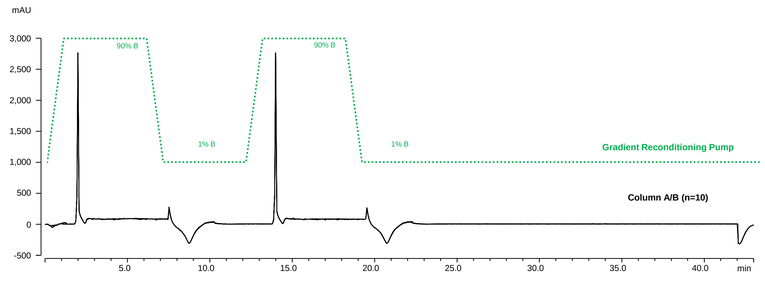
The average absolute retention time shift between column A and column B was 0.023 min (relative, 0.18 %) and shows that peak assignment based on retention time is not impaired. An average peak area RSD value of 2.47% demonstrates the suitability for quantitative data analysis using the tandem LC setup.

Table 3. Reproducible results for the Vanquish Duo System for Tandem LC - Q Exactive HF MS 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 5.

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**	**2.593**		**0.039**	**2.138**	**0.023**	**0.18**	**0.11**	**2.472**

The advanced wash and reconditioning method used 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 6).

Figure 6. Overlay of ten chromatograms of the tandem LC reconditioning step.



CONCLUSIONS

- The Vanquish Horizon Duo UHPLC system for Tandem LC-MS workflows 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 7.2.8 and Thermo Scientific™ SII for Xcalibur™ version 1.4 support the Vanquish Duo Tandem LC-MS workflows.

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2. Van Middlesworth, B. J.; Dorsey, J. G., Re-equilibration Time of Superficially Porous Silica Based Columns in Gradient Elution Reversed Phase Liquid Chromatography. *J. Chromatogr., 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.

TRADEMARKS/LICENSING

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