High-throughput peptide mapping of trastuzumab using a tandem LC-MS workflow

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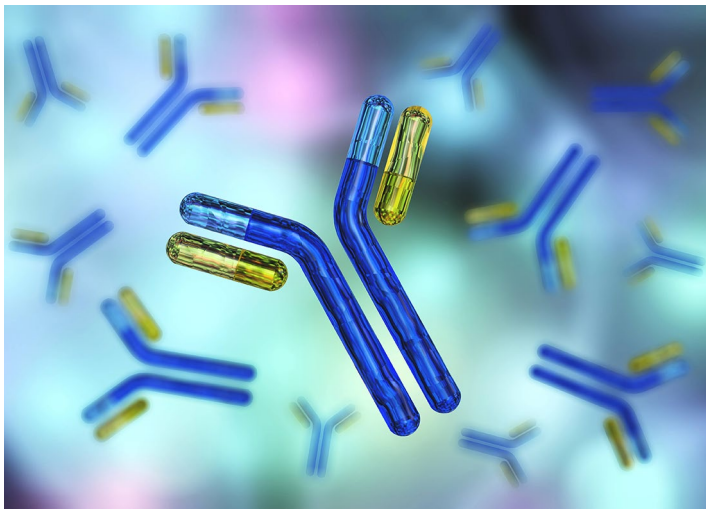
Key benefits

- Operational simplicity of tandem LC-MS methods setup using Thermo Scientific™ Chromeleon™ CDS with compliance-ready enterprise acquisition of LC-MS data for cGMP environments.
- High-throughput peptide mapping of biopharmaceuticals with at least 25% reduction in analysis time when compared to standard LC operation.
- Tandem LC-MS workflow eliminates MS standby time.
- Excellent column to column retention time reproducibility and PTMs for analysis performed on two separate columns in tandem mode.

Goal

- Demonstrate easy setup of Thermo Scientific™ Vanquish™ Duo UHPLC system for Tandem LC-MS workflow and seamless creation of the instrument methods using Chromeleon CDS.



- Demonstrate time-savings, without loss of instrument performance for peptide mapping analysis of monoclonal antibodies.
- Tandem LC-MS workflow to eliminate MS instrument idle time.

Introduction

Therapeutic protein development and production requires high-throughput sample analysis and high-quality data. Orthogonal analyses of protein PTMs are required to ensure that critical quality attributes (CQAs) are within the established ranges, to maintain lot-to-lot consistency in line with regulatory requirements and ICH guidelines.[1] Peptide mapping is considered a golden standard for monoclonal antibody (mAb) analysis as it provides information on many attributes within a single analysis. The recently described Multi-Attribute Method (MAM) is based on

peptide mapping LC-MS analysis and allows high quality, confident PTM identification in QC laboratories.[2-4] However, a disadvantage of this technique is the long analysis time required, due to the lengthy LC gradient that is necessary to resolve peptides in the complex tryptic mixture. Moreover, column washing and re-equilibration steps between subsequent analyses generate a lag where the MS instrument is idle and eluents are usually diverted to waste. Innovations within UHPLC instrumentation have led to the development of a dual LC system that can achieve high-throughput "tandem-mode" LC-MS analysis, with no loss of data quality or resolution. The Vanquish Duo system for Tandem LC-MS workflows accomplishes tandem-mode LC-MS by performing the analysis on two pumps and two columns all in one system (Figure 1).

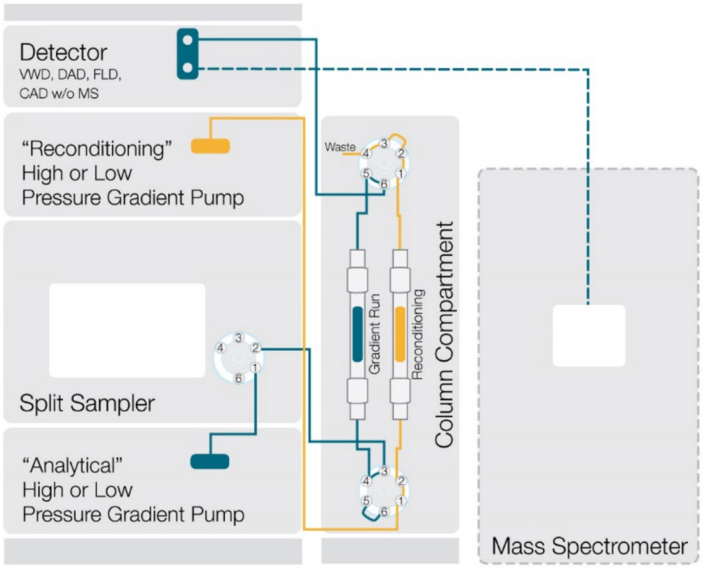


Figure 1. Vanquish Duo for Tandem LC-MS plumbing scheme

The system delivers an analytical gradient on the first pump, while the second pump washes and reconditions a second column, preparing it for the next injection (Figures 1 and 2). The sequences alternate between the two columns. When the separation gradient on column 1 is complete, position switching valves in the column compartment before and after the LC columns divert the analytical pump to column 2, starting a new analysis while washing of column 1 starts and re-equilibration steps are performed offline, thus MS idle time is minimized (Figure 2).

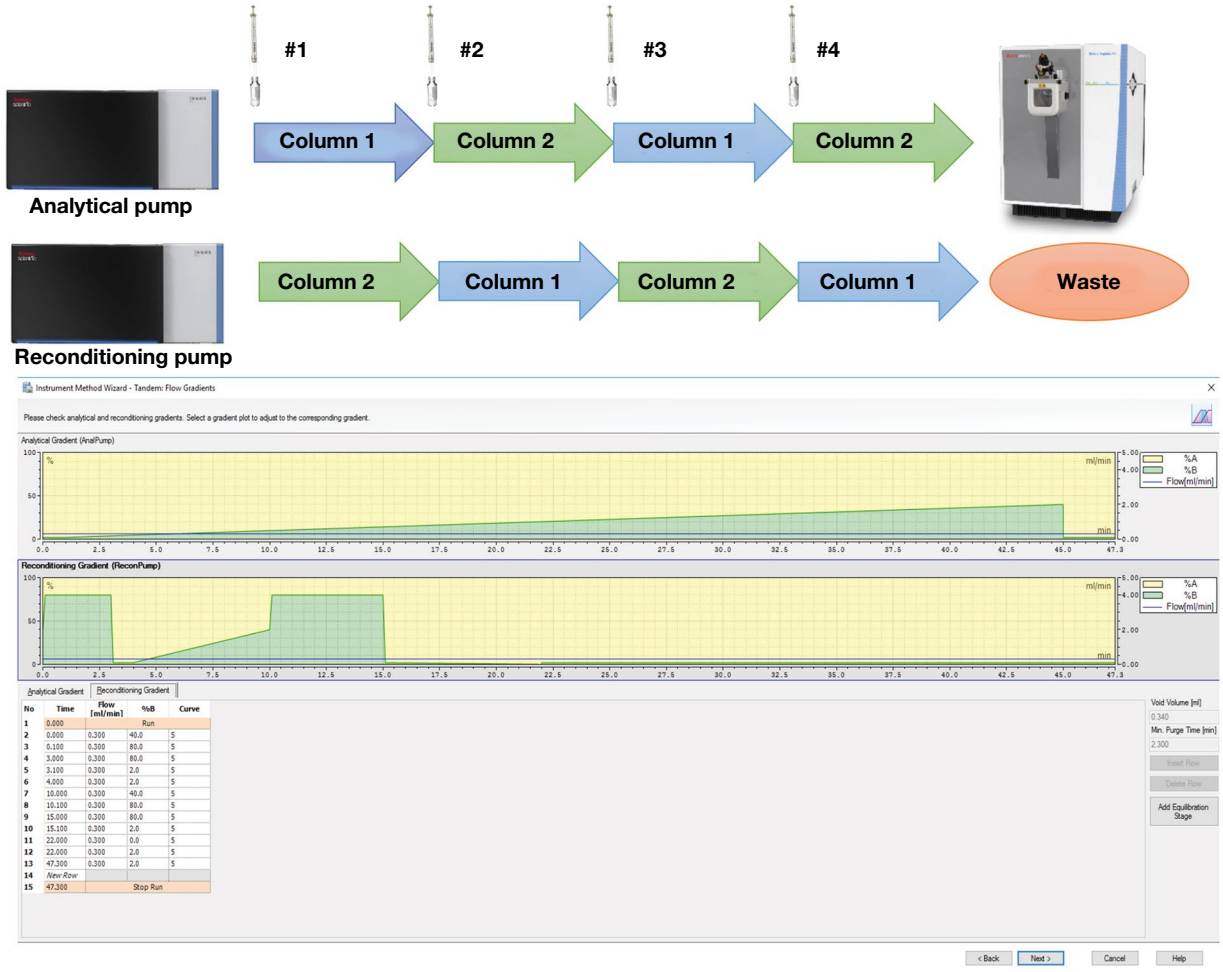


Figure 2. Schematic representation of Tandem LC-MS workflow and sequence setup in Chromeleon CDS

Here, we demonstrate the applicability of the Vanquish Duo UHPLC system for Tandem LC-MS workflow and a Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer for peptide mapping analysis of trastuzumab drug product. With this approach, a greater number of sample injections can be performed in the same timeframe, enhancing throughput and significantly reducing MS idle time (Figure 2).

Chromatographic results proved to be equivalent across the two chromatographic channels. Moreover, the reproducibility of PTM values was assessed during a continuous 16 h period of operation, proving the stability of MS performance under continuous acquisition.

Experimental

Sample preparation

- Thermo Scientific™ SMART Digest™ Trypsin kits, Magnetic Bulk Resin option (P/N 60109-101-MB)
- Thermo Scientific™ KingFisher™ Duo Prime system (P/N 5400100)
- Thermo Scientific™ PP Screw vials and caps (P/N 03-PPSVW, 9-SCK(B)-ST1)

Samples were prepared following an earlier published method*.

Chromatography

System

- Vanquish Horizon Duo UHPLC System consisting of:
  - System Base Vanquish Flex/Horizon (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)
  - Workflow Kit, Vanquish Duo for Tandem LC (P/N 6036.2020)

Column

- Thermo Scientific™ Acclaim™ VANQUISH™ C18 2.1 × 250 mm, 2.2 µm (P/N 074812-V)

Solvents

- A) Water with 0.1% formic acid (v/v), Optima™ LC/MS grade, Fisher Chemical™ (P/N 10188164)
- B) Acetonitrile with 0.1% formic acid (v/v), Optima™ LC/MS grade, Fisher Chemical™ (P/N 10118464)

Gradient

- Flow 0.3 mL/min
- Gradient details, see Figure 2

Mass spectrometry

Instrument

- Orbitrap Exploris 240 (P/N BRE725535) with BioPharma Option (P/N BRE725539)

MS acquisition

- Resolution 120,000 FWHM

Application-specific MS tune and acquisition settings are templated and provided within the software. Settings are directly transferable from instrument to instrument, enabling easy method transfer and operational simplicity.

Software

- Chromeleon CDS version 7.2.10
- Thermo Scientific™ BioPharma Finder™ Software 4.0 (P/N OPTON-30985)

Results and discussion

Peptide mapping is a routine analysis in biopharmaceutical assessment and is gaining more interest as part of the multi-attribute method (MAM) workflow driving the establishment of mass spectrometry in the QC laboratory. Method transfer in the QC environment requires improved robustness and high throughput due to the increasing number of samples that need to be processed. In this study we prove the suitability of Vanquish Duo UHPLC system for peptide mapping analysis and for the MAM workflow, reducing the time window where LC needs to be diverted to waste and the mass spectrometer is idle.

Trastuzumab drug product was digested using the Smart Digest trypsin kit magnetic option and the generation of tryptic peptides was performed automatically on the KingFisher Duo Prime system. The digested monoclonal antibody was then analyzed via Tandem LC-MS/MS analysis. Chromeleon CDS software automatically splits the gradient across the two columns and pumps; when

* Millán-Martín, S.; Jakes, C.; Carillo, S. *et al.* Inter-laboratory study of an optimised peptide mapping workflow using automated trypsin digestion for monitoring monoclonal antibody product quality attributes. *Anal. Bioanal. Chem.* **(2020)**. https://doi.org/10.1007/s00216-020-02809-z

the analytical gradient is complete, the wash step starts. Twenty-one injections were performed, alternating on the two Acclaim VANQUISH C18 2.1 × 250 mm columns used in this study, to obtain a perfect overlap of data coming from the two channels (Figure 3).

Firstly, chromatographic reproducibility was evaluated across the gradient by plotting the retention times for five different peptides along with the 21 injections. No difference was observed between the two data sets, returning overall relative standard deviation (RSD) values lower than 1.5% (Figure 4). Excellent reproducibility in the retention time is important for a correct identification of MS components to evaluate and quantify drug product CQAs. However, it would be possible to allow higher values of %RSD for the retention times as the use of high-resolution MS gives a confident identification of MS components even when using a wider retention time window.

Peptide mapping analysis is mainly performed to evaluate PTMs in therapeutic proteins that need to be monitored during bioprocessing and batch release to guarantee drug product safety. We evaluated product quality attributes for trastuzumab across the 21 injections performed in tandem mode (Table 1) using BioPharma Finder software. Each modification was calculated considering up to one missed cleavage and without considering Na^+ and K^+ adducts or non-specific cleavages, using a confidence score ≥ 95% and within ± 5 ppm. Detection of most PTMs resulted in very low RSD values, that are exceeding 15% only for some low abundant modifications (N328 + deamidation = 0.20%), proving excellent chromatographic equivalence of the two channels for PTM evaluation.

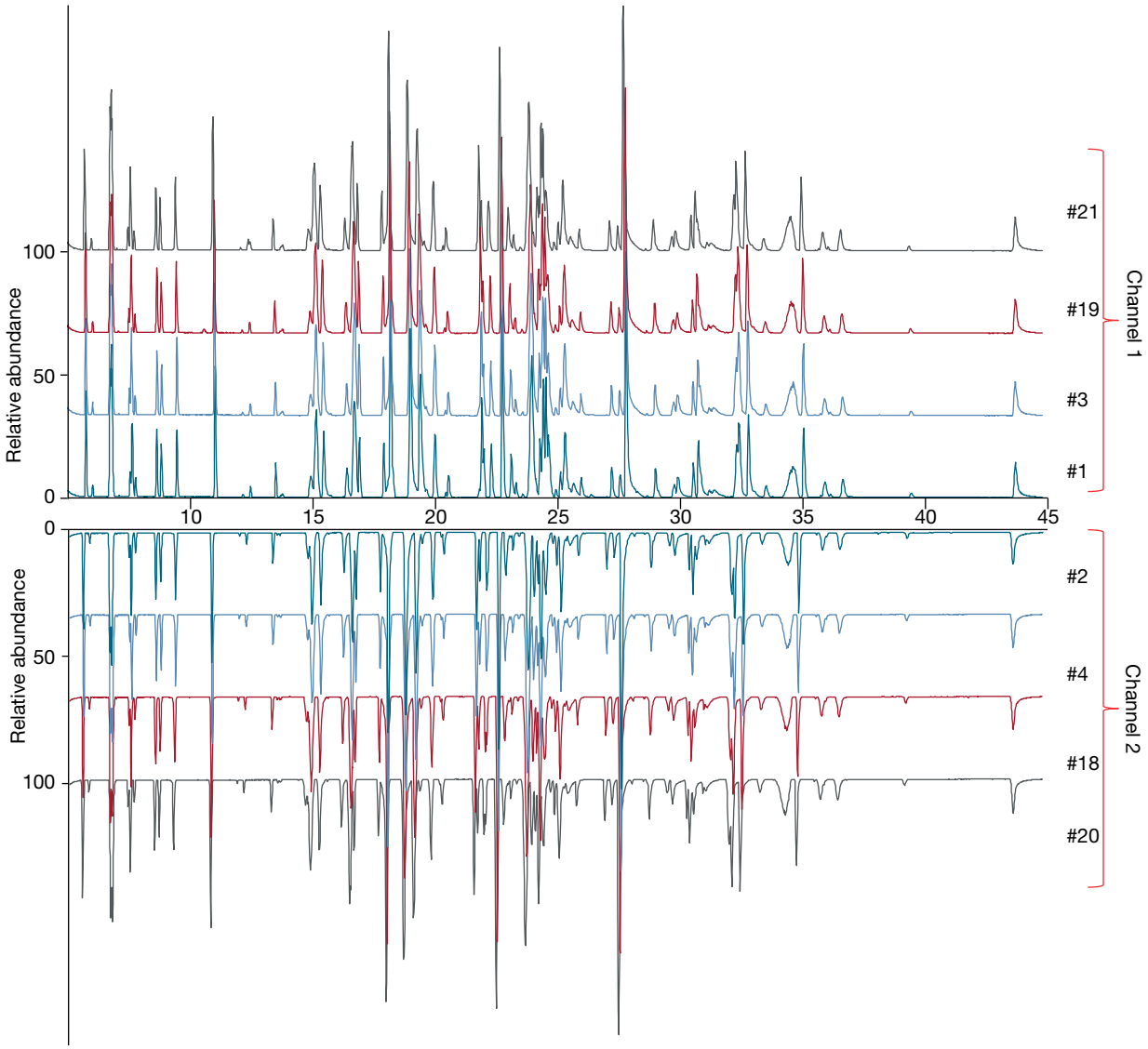


Figure 3. Mirror plot of the overlays of trastuzumab peptide mapping Full MS base peak chromatograms obtained on column 1 (top) and column 2 (bottom)

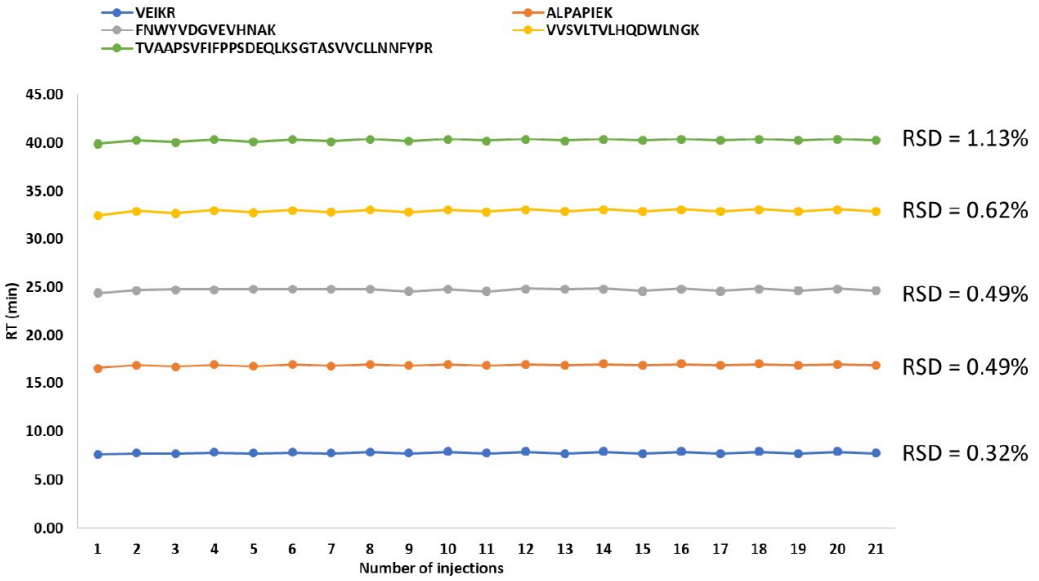


Figure 4. Trend of the retention time from XIC of individual charge states for VEIKR, FNWYVDGVEVHNAK, TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPR, ALPAPIEK, and VVSVLTVLHQDWLNGK tryptic peptides across 21 injections performed in tandem mode on the Vanquish Duo UHPLC system

Table 1. Trastuzumab drug product quality attributes

Subunit	Modification	% Abundance Avg (n=21)	RSD [%] (n=21)
HC	HC N55+Deamidation	2.44	2.82
HC	HC N77+Deamidation	0.55	3.64
HC	HC N289+Deamidation	0.08	8.18
HC	HC N318+Deamidation	1.11	12.72
HC	HC N328+Deamidation	0.20	19.65
HC	HC N364+Deamidation	0.85	8.08
LC	LC N30+Deamidation	6.18	3.71
HC	Q39+NH_3 loss/Succ	3.38	3.48
HC	N55+NH_3 loss/Succ	3.37	3.89
HC	N289+NH_3 loss/Succ	0.12	6.35
HC	N318+NH_3 loss/Succ	5.12	4.06
HC	~N387+NH_3 loss/Succ	0.62	5.87
HC	M83+Oxidation	0.05	13.79
HC	M255+Oxidation	0.98	8.05
HC	M361+Oxidation	1.07	14.73
LC	M4+Oxidation	0.08	9.65
HC	D102+H_2O loss/Succ	1.27	6.04
HC	~D283+H_2O loss/Succ	1.51	5.86
HC	~D402+H_2O loss/Succ	1.51	8.20
LC	~D17+H_2O loss/Succ	0.23	13.84
HC	K30+Glycation	0.35	6.15
HC	K136+Glycation	0.17	4.65
HC	K320+Glycation	0.05	9.07
HC	K329+Glycation	0.74	7.01
HC	D62+Isomerization	0.24	5.94
HC	E1+H_2O loss (PyroE)	0.98	6.99
HC	G449+Lys	2.13	5.60
HC	N300+A1G0	1.74	3.31
HC	N300+A1G0F	3.66	4.58
HC	N300+A1G0M4F	0.72	4.59
HC	N300+A2G0	5.78	1.47
HC	N300+A2G0F	48.03	0.73
HC	N300+A2G1F	32.03	1.19
HC	N300+A2G2F	4.34	1.84
HC	N300+M5	3.05	3.13

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Conclusion

Injection of 21 samples of trastuzumab drug product tryptic digest were successfully analyzed by LC-MS/MS analysis in tandem mode on the Vanquish Duo UHPLC system for Tandem LC-MS and Orbitrap Exploris 240 MS platform. System performance was evaluated by monitoring retention time reproducibility and identified PTMs, with both evaluations yielding excellent data and low %RSD values. Tandem analysis of peptide mapping replicates did not affect the accuracy of the results and the correct identification, saving at least 6 h of instrument time when compared to single mode during 24 h. The utilization of SMARTDigest Kits versus in-solution digestion has already been shown to save between 2–20 hours preparation time per sample.[5] Adding sample preparation time savings to significantly increased throughput from the instrumental analysis demonstrated here makes this entire workflow a highly productive alternative to traditional methods, while maintaining or improving performance and reproducibility. In conclusion, the Vanquish Duo UHPLC system represents a powerful instrument to increase laboratory productivity without compromising data quality, especially when combined with automated sample preparation of the tryptic digest.

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