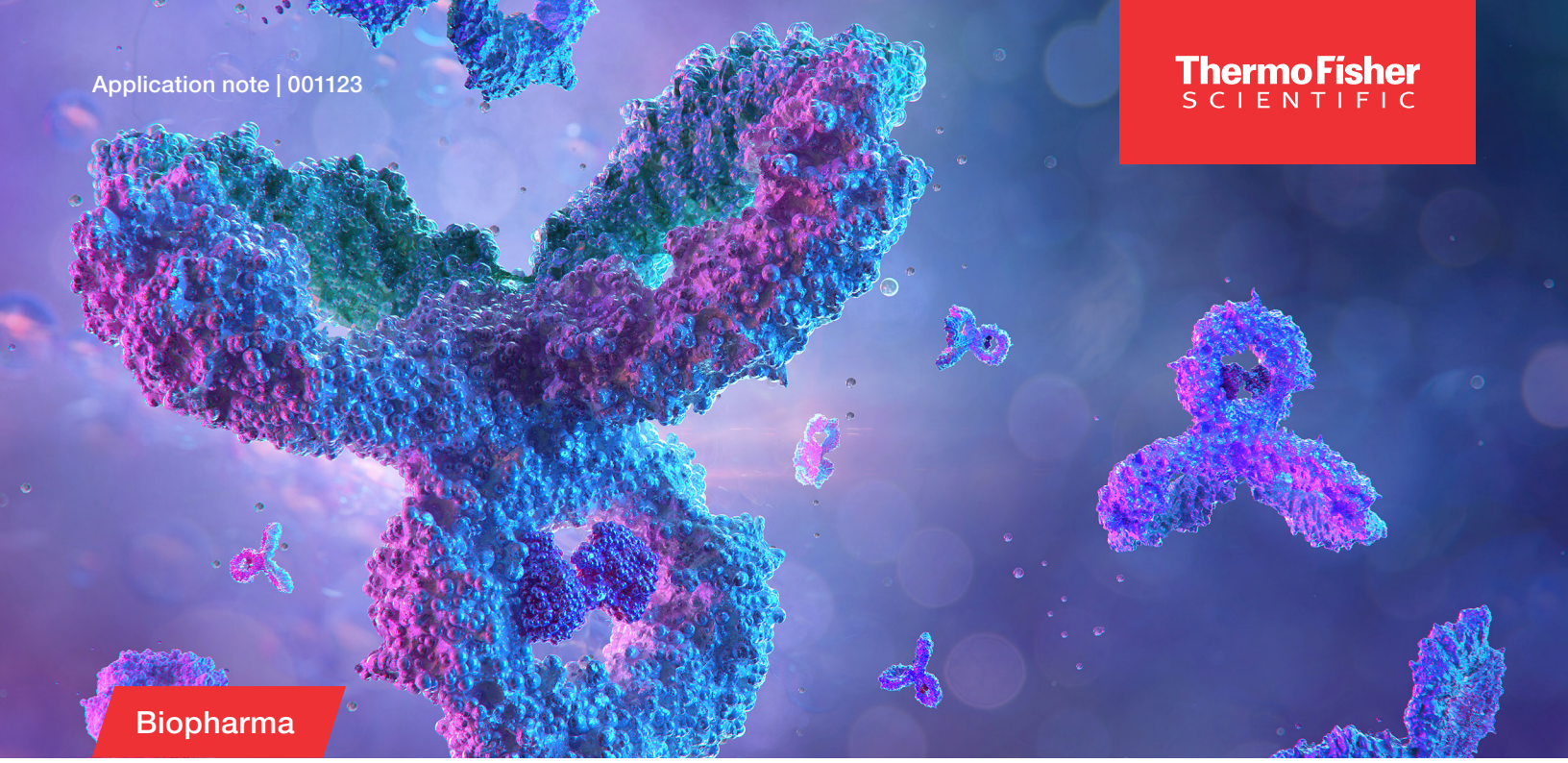


# Consistent results for peptide mapping and monitoring across three systems of the Vanquish UHPLC platform


## Authors

Craig Jakes[1,2], Silvia Millán-Martín[1], Sara Carillo[1], Dennis Köhler[3], Frank Steiner[3], Kai Scheffler[3], Jonathan Bones[1,2]

[1]NIBRT, Dublin, Ireland

[2]School of Chemical Engineering, UCD, Dublin, Ireland

[3]Thermo Fisher Scientific, Germering, Germany



## Keywords

Vanquish Flex UHPLC, Q Exactive Plus MS, Vanquish Horizon UHPLC, Vanquish Tandem LC system, HPLC, method development, method transfer, gradient delay volume, multi attribute method, AQbD, Chromeleon CDS


## Goal

Assessing consistency, reproducibility of results, and method transferability across the Thermo Scientific™ Vanquish™ Horizon UHPLC, Thermo Scientific™ Vanquish™ Flex UHPLC, and Thermo Scientific™ Vanquish™ Tandem LC systems based on two protein digest samples applying a 30 min and a 90 min gradient.

## Application benefits

- The Vanquish Horizon, Vanquish Flex, and Vanquish Tandem LC systems provide highly consistent and reproducible results with exceptional precision for qualitative and quantitative peptide mapping and peptide monitoring.
- Chromatographic methods are transferable across all systems upon consideration of gradient length.
- The Vanquish Tandem LC system supports maximizing efficiency of the Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap™ MS and increasing throughput. Here, we achieved 46% and 23% increases.

## Introduction

Ultra-high performance liquid chromatography coupled to high-resolution, accurate-mass mass spectrometry (UHPLC-HRAM-Orbitrap-MS)-based peptide mapping and monitoring are the most commonly applied tools for the analysis of protein-based therapeutics at multiple stages throughout the development cycle.

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While in the characterization phase, the goal of the analysis is to fully characterize the molecule under study. MS/MS spectra are essential to derive all peptide amino acid sequences as well as the type and location of any modifications. Yet, in the routine analysis in later stages of the development cycle, the combination of retention time with accurate mass information is typically sufficient to confidently identify all peptides to assess sequence coverage or alternatively monitor a selected set of peptides. In either case, reliability and reproducibility of retention times are key in achieving high confidence results. The capability of the LC pump to deliver the mobile phase composition in a highly consistent manner is one of the major factors for achieving reproducible results on a particular chromatography system and a requirement for successful method transfer across labs and across organizations. When comparing results from different models of a chromatography platform or even different platforms, additional factors come into play. One of which is the gradient delay volume (GDV) that is defined as the volume between the point of mobile phase mixing and the column entry, resulting from a combination of volumes contributed by pumping system, gradient mixer, tubing between the pump and the injector, injector loop and metering device, and tubing between the injector and the column.[1,2]

Here, we aimed to compare three systems from the Thermo Scientific Vanquish UHPLC platform: The Vanquish Horizon system, the Vanquish Flex Binary system, and the Vanquish Tandem LC system, comprising low pressure gradient (LPG) Vanquish Flex binary pumps for running two identical columns in tandem operation.[3,4] There are several commonalities amongst all three systems, such as biocompatibility, choice of add-on detectors, and autosampler injection volumes from 0.01 to 100 µL, but there are also several differentiators. The Vanquish Horizon UHPLC has the highest pressure limit of 1,500 bar, allowing for the most demanding method setup with high flow rates even for columns with very small particle size, supporting increased sample throughput. The Vanquish Flex system, designed for application flexibility, supports method scouting, in particular in the setup with the quaternary pump, and routine analyses for flow rates up to 8 mL/min with a pressure limit of 1,000 bar. The Vanquish Tandem LC system supports the increase in throughput by having one column in the flow path only for the duration of running the analytical gradient, while the second column is being regenerated and equilibrated offline, thus maximizing the use of the mass spectrometer for detection.[3,4]

In this study, we have used the chromatography systems in their standard configurations to evaluate the comparability of retention times and method transferability using a bovine serum albumin (BSA) digest sample separated across a 30 min gradient and a NISTmAb digest sample separated via a 90 min gradient on all three systems. Each data set consisted of ten replicate injections from each sample on every system and on each of the two columns run on the Vanquish Tandem LC system. In addition, two time points were collected two weeks apart. The scope of the experimental plan was to perform the overall data analysis by monitoring a set of modified and unmodified peptides to assess reproducibility and precision of quantitative levels of peptides and their modifications using the Thermo Scientific™ Chromeleon™ Chromatography Data System based on data acquired on a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer. An initial data set for both samples was acquired including MS/MS spectra for the analysis in Thermo Scientific™ BioPharma Finder™ software to establish a peptide target workbook. This was imported by Chromeleon software as a basis for data processing of Full MS only mass spectrometry data, targeting the selected set of relevant peptides. This Multi-Attribute Method (MAM) type approach was applied as it is a more thorough evaluation of results compared to a mere comparison of retention times and peak intensities. An overview of the experimental setup is presented in Figure 1.

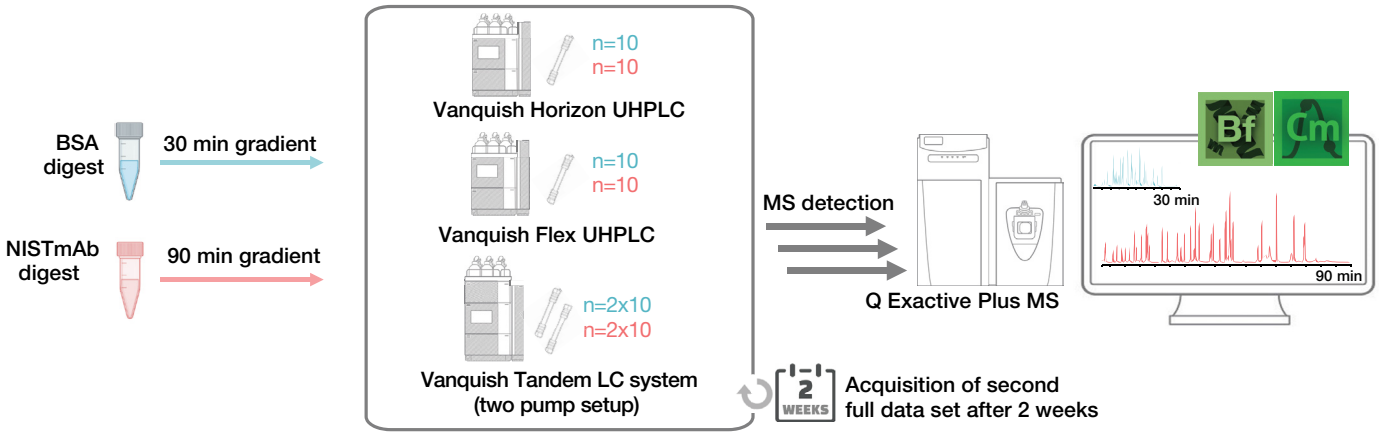



**Figure 1. Overview of the experimental setup applied in this study**

## Experimental

### Reagents and consumables

- NISTmAb Humanized IgG1κ Monoclonal Antibody Lot 14HD-D002 (NIST, RM 8671)
- BSA Protein Digest, MS grade, Thermo Scientific™ Pierce™ (P/N 88341)
- 8.0 M Guanidine hydrochloride solution (Sigma, P/N G7294-100ML)
- Invitrogen™ UltraPure™ 1 M Tris-HCl Buffer, pH 7.5 (P/N 15567027)
- Sodium hydroxide concentrate (Sigma, P/N 43617-1L)
- Sodium iodoacetate (IAC), BioUltra >98% purity (Sigma, P/N I-9148)
- DL-Dithiothreitol (DTT), BioXtra ≥99% purity (Sigma, P/N D-5545)
- Formic acid, LC-MS grade, Thermo Scientific™ Pierce™ (P/N 28905)
- Trypsin protease MS grade, Thermo Scientific™ Pierce™ (P/N 90058)
- Bio-Spin™ P-6 Gel Columns, Tris Buffer (Bio-Rad, P/N 732-6227)
- Eppendorf™ Protein LoBind Microcentrifuge Tube 0.5 mL (P/N 022431064)
- Eppendorf™ Protein LoBind Microcentrifuge Tube 1.5 mL (P/N 022431081)
- Thermo Scientific™ Acclaim™ VANQUISH™ UHPLC column, 2.1 × 250 mm, 2.2 µm (P/N 074812-V)
- Water, UHPLC-MS grade, Thermo Scientific™ (P/N W8-1)
- Acetonitrile, UHPLC-MS grade, Thermo Scientific™ (P/N A956-1)
- Methanol, UHPLC-MS grade, Thermo Scientific™ (P/N A458-1)
- Formic acid (FA), 99.0%, Optima™ LC-MS grade, Thermo Scientific™ (P/N 10797488)
- Screw vial caps and seals, Thermo Scientific™ (P/N 10656984)
- 9 mm glass autosampler inserts, 400 µL, Thermo Scientific™ (P/N 11911563)

### Sample preparation

For BSA, no sample preparation was required as it was obtained as a predigested, lyophilized sample. For reconstitution, 1 mL of 0.1% formic acid was added to the original glass container, which was vigorously vortexed for 15 s before transferring 100 µL into an autosampler vial. The residual sample was immediately stored at -20 °C.

For NISTmAb, samples were prepared and digested using the following stock reagents:

#### Solution 1: 7.0 M Guanidine HCl, 100 mM Tris (pH 8.3)

87.5 mL of 8 M guanidine HCl and 10 mL of 1 M Tris-HCl pH 7.5 were combined followed by pH adjustment to a pH of 8.3 with sodium hydroxide concentrate. LC-MS grade water was added to bring the total volume to 100 mL.

#### Solution 2: 500 mM DTT in solution 1

50 mg of DTT was freshly weighed into a 1.5 mL Eppendorf tube and 649 µL of solution 1 was added. The solution was mixed by vortex until the solution became clear.

#### Solution 3: 500 mM IAC in solution 1

50 mg of IAC was freshly weighed into a 1.5 mL Eppendorf tube and 481 µL of solution 1 was added. The solution was mixed by vortex until the solution became clear. It is important to store solution 3 in the absence of light.

#### Solution 4: 50 mM DTT in solution 1

100 µL of solution 2 was added to 900 µL of solution 1 in a fresh 1.5 mL Eppendorf tube and mixed thoroughly by vortex.

#### Solution 5: 50 mM Tris (pH 7.9)

10 mL of 1 M Tris-HCl pH 7.5 was added to a 200 mL volumetric flask and brought to a final volume of 200 mL using LC-MS grade water. The flask was mixed through inversion and the pH was verified using a pH meter.

#### Solution 6: 10% Formic acid

1 mL of LC-MS grade formic acid was added to 9 mL of LC-MS grade water in a 15 mL tube and mixed by vortex.

### Sample reduction and alkylation

Ten aliquots containing 100 µg each of NISTmAb were diluted to 1 mg/mL with solution 1 to give a final volume of 100 µL. 2 µL of solution 2 was added to the samples and mixed by vortex. Reduction was carried out by allowing the samples to stand at room temperature for 30 minutes. 4 µL of solution 3 was added to each sample and mixed by vortex. Alkylation was carried out by allowing the samples to stand at room temperature for 20 minutes in the absence of light. Alkylation was quenched by adding 4 µL of solution 4 to each sample and mixed by vortex.

### Buffer exchange

BioSpin-6 columns were conditioned first by breaking off the tip and inserting the bottom of the column into a 2 mL collection tube. The columns were centrifuged at 1,000 × g for 2 minutes and the flow through was discarded. 500 µL of solution 5 was gently pipetted to the bed of the BioSpin-6 column and centrifuged at 1,000 x g for 2 minutes. The flow through was discarded. This step was repeated three more times for a total of 4 washes. Following the washing procedure, the conditioned column was placed in a fresh 1.5 mL LoBind Eppendorf tube. 110 µL of the reduced and alkylated samples were added to the bed of the column. The columns were then centrifuged at 1000 × g for 4 minutes and the flow through was collected.

### Sample digestion

Pierce trypsin protease was reconstituted by adding 100 µL of LC-MS grade water to the vial. 10 µL of trypsin was added to 100 µL of buffer exchanged mAb samples with a ratio of 1:10 v/v. The samples were mixed briefly by vortex before incubation at 37 °C for 30 minutes. 11 µL of solution 6 was added to the samples post digestion to quench any residual trypsin and was briefly mixed by vortex. All digested samples were pooled and then aliquoted again in HPLC vials for the analysis. Aliquots not in use were kept at -80 °C.

### Chromatography

#### Column assessment and column use

For all experiments in this study, two Acclaim VANQUISH C18 2.1 × 250 mm, 2.2 µm columns were used. Column conditioning was performed by exposure of the columns to 70% ACN for 1 hour.

As alignment in column performance is crucial in particular for the Vanquish Tandem LC system, the two columns used were evaluated prior to performing the experiments using a column test based on a mixture of uracil (15 mg), phenanthrene (15 mg), and dimethyl phthalate (75 µL), dissolved in 50 mL 70% acetonitrile/30% water in a clean, dry media bottle. Upon adding another 50 mL of 70% acetonitrile/30% water, the solution was sonicated before analysis. Six replicate runs on each column were performed injecting 1 µL of the mixture and separating the compounds using an isocratic flow of 70% ACN/30% water at a flow rate of 0.4 mL/min with UV detection at 254 nm. Column performance was assessed based on retention time, peak shape, peak height, and obtained back pressure.

In order to maintain the same column aging for the actual experiments, columns 1 and 2 were first used for parallel analysis on the Vanquish Tandem LC system, then one column each was dedicated to BSA and NISTmAb analysis, respectively, on the other instruments before being employed for the Tandem analysis again after two weeks. With this column use, the results obtained in particular for the Vanquish Horizon and Vanquish Flex systems were obtained on the exact same columns and could thus be excluded as a possible source for any observed variations.

#### Chromatography systems

Three Vanquish UHPLC systems were used in this study. The modules included in each system are listed in Table 1. Applied gradients are detailed for each UHPLC system in Table 2. Unless otherwise stated, 5 µL containing a total of 5 µg digest samples were injected using the LC gradient and conditions outlined in Table 2 and Table 3 for BSA protein digest and NISTmAb digested samples, respectively.

**Table 1. Vanquish UHPLC system modules and part numbers**

| Module | Vanquish Horizon | Vanquish Flex Binary | Vanquish Duo (two binary pump setup) |
|---|---|---|---|
| **System Base** | VF-S01-A | VF-S01-A | VF-S01-A |
| **Pump 1** | VH-P10-A | VF-P10-A | VF-P10-A |
| **Pump 2** | N/A | N/A | VF-P10-A |
| **Split Sampler** | VH-A10-A | VF-A10-A | VF-A10-A |
| **Column Compartment** | VH-C10-A | VH-C10-A | VH-C10-A |
| **Thermo Scientific™ Viper™ Capillary Kit** | N/A | N/A | Viper Kit for Vanquish Tandem LC system, P/N 6036.2020 |

**Table 2. LC and autosampler conditions for separation of BSA peptides**

| Parameter | Vanquish Horizon | Vanquish Flex | Vanquish Tandem |
|---|---|---|---|
| Column temp. | 25 °C | 25 °C | 25 °C |
| Flow rate | 0.3 mL/min | 0.3 mL/min | 0.3 mL/min |
| Solvent A | $H_2O$ + 0.1% FA | $H_2O$ + 0.1% FA | $H_2O$ + 0.1% FA |
| Solvent B | ACN + 0.1% FA | ACN + 0.1% FA | ACN + 0.1% FA |
| Gradient | Time (min) %B<br>0.0 2.0<br>30.0 40.0<br>30.05 80.0<br>34.0 80.0<br>34.5 2.0<br>40.0 40.0<br>40.5 80.0<br>44.0 80.0<br>44.5 2.0<br>60.0 2.0 | Time (min) %B<br>0.0 2.0<br>30.0 40.0<br>30.5 80.0<br>34.0 80.0<br>34.5 2.0<br>40.0 40.0<br>40.5 80.0<br>44.0 80.0<br>44.5 2.0<br>60.0 2.0 | Time (min) %B<br>Analytical<br>0.0 2.0<br>30.0 40.0<br>30.0 2.0<br>32.7 2.0<br>Reconditioning<br>0.0 40.0<br>0.5 80.0<br>4.0 80.0<br>4.5 2.0<br>10.0 40.0<br>10.5 80.0<br>16.0 80.0<br>16.5 2.0<br>32.7 2.0 |
| Injection volume | 5 µL | 5 µL | 5 µL |
| Needle wash | 10% MeOH with 0.1% FA (after draw, before injection) | 10% MeOH with 0.1% FA (after draw, before injection) | 10% MeOH with 0.1% FA (after draw, before injection) |
| Seal rise solution | 10% MeOH | 10% MeOH | 10% MeOH |
| Autosampler temp. | 6 °C | 6 °C | 6 °C |
| Thermostatting mode | Still Air | Still Air | Still Air |
| Pre-heater | Not used | Not used | Not used |
| Wash speed/time | 30 µL/s for 10 s | 30 µL/s for 10 s | 30 µL/s for 10 s |

**Table 3. LC and autosampler conditions for separation of NISTmAb peptides**

| Parameter | Vanquish Horizon | Vanquish Flex | Vanquish Tandem |
|---|---|---|---|
| Column temp. | 25 °C | 25 °C | 25 °C |
| Flow rate | 0.3 mL/min | 0.3 mL/min | 0.3 mL/min |
| Solvent A | $H_2O$ + 0.1% FA | $H_2O$ + 0.1% FA | $H_2O$ + 0.1% FA |
| Solvent B | ACN + 0.1% FA | ACN + 0.1% FA | ACN + 0.1% FA |
| Gradient | Time (min) %B<br>0.0 2.0<br>90.0 40.0<br>90.05 80.0<br>94.0 80.0<br>94.5 2.0<br>100.0 40.0<br>100.5 80.0<br>104.0 80.0<br>104.5 2.0<br>120.0 2.0 | Time (min) %B<br>0.0 2.0<br>90.0 40.0<br>90.5 80.0<br>94.0 80.0<br>94.5 2.0<br>100.0 40.0<br>100.5 80.0<br>104.0 80.0<br>104.5 2.0<br>120.0 2.0 | Time (min) %B<br>Analytical<br>0.0 2.0<br>90.0 40.0<br>90.0 2.0<br>92.7 2.0<br>Reconditioning<br>0.0 40.0<br>0.5 80.0<br>4.0 80.0<br>4.5 2.0<br>10.0 40.0<br>10.5 80.0<br>20.0 80.0<br>20.5 2.0<br>92.7 2.0 |
| Injection volume | 5 µL | 5 µL | 5 µL |
| Needle wash | 10% MeOH with 0.1% FA (after draw, before injection) | 10% MeOH with 0.1% FA (after draw, before injection) | 10% MeOH with 0.1% FA (after draw, before injection) |
| Seal rise solution | 10% MeOH | 10% MeOH | 10% MeOH |
| Autosampler temp. | 6 °C | 6 °C | 6 °C |
| Thermostatting mode | Still Air | Still Air | Still Air |
| Pre-heater | Not used | Not used | Not used |
| Wash speed/time | 30 µL/s for 10 s | 30 µL/s for 10 s | 30 µL/s for 10 s |

## Mass spectrometry

For peptide mapping analysis, BSA digest and NISTmAb peptides were analyzed on Vanquish Tandem LC system coupled to a Q Exactive Plus mass spectrometer using a data-dependent acquisition (DDA) MS/MS method. For the targeted peptide monitoring of selected peptides and CQAs, a full MS scan method was used for mass detection in combination with all three UHPLC systems. Detailed instrument methods and source parameters for the mass spectrometer are summarized in Table 4.

## Data processing with BioPharma Finder software for sequence coverage assessment and workbook creation

Peptide identification and CQAs assessment were performed using BioPharma Finder software version 4.1, according to the parameters summarized in Table 5. A target peptide workbook was created for both BSA and NISTmAb samples, containing a list of peptides to monitor and the most prominent CQAs, including all the detected charge states. The results were selected to include components with up to 1 missed cleavage. Moreover, peptides containing $Na^+/K^+$ adducts were excluded together with nonspecific, unknown modifications and gas phase ions. The target peptide workbook contains information about the selected target peptides that will be used to run a targeted peptide monitoring analysis by exporting the data to a file (.wbpf) compatible with the Chromeleon software.

**Table 4. Instrument method parameters for the Q Exactive Plus Orbitrap mass spectrometer**

| Source parameters (HESI-II) | |
|---|---|
| Spray voltage (V) | 3,800 |
| Sheath gas (Arb) | 40 |
| Aux gas (Arb) | 10 |
| Capillary temperature (°C) | 320 |
| Probe heater temperature (°C) | 400 |
| S-lens RF voltage (V) | 50 |
| **Full MS scan settings** | |
| Full MS mass range *(m/z)* | 200–2,000 |
| Resolution setting | 140,000 |
| Target value | 3e6 |
| Max. injection time (ms) | 100 |
| Default charge state | 2 |
| **$MS^2$ parameters** | |
| Resolution setting | 17,500 |
| Target value | 5e5 |
| Isolation width *(m/z)* | 1.2 |
| Signal threshold | 1e4 |
| Normalized collision energy (HCD) | 28 |
| TopN $MS^2$ | 5 |
| Max. injection time (ms) | 200 |
| Dynamic exclusion (s) | 7 |

**Table 5A. BioPharma Finder software parameter settings for peptide mapping data analysis**

| Component detection | Setting |
|---|---|
| **Absolute MS signal threshold (counts)** | $2.0 \times 10^4$ |
| **Typical chromatographic peak width** | 0.3 |
| **Mass tolerance (ppm)** | 4.0 |
| **Maximum retention time shift (min)** | 0.5 |
| **Maximum mass (Da)** | 30,000 |
| **Identification** | **Setting** |
| **Max. peptide mass** | 7,000 |
| **Mass accuracy (ppm)** | 8 |
| **Min. confidence** | 0.8 |
| **Max. # modifications** | 1 |
| **Protease specificity** | High |
| **N-glycosylation** | CHO (NISTmAb)<br>None (BSA) |

**Table 5B. BioPharma Finder software parameter settings for peptide mapping data analysis (modifications)**

| Modification | Parameter |
|---|---|
| **Static** | Carboxymethylation (NISTmAb)<br>Carbamidomethylation (BSA) |
| **Variable** | **NISTmAb**<br>N-term PyroGlu<br>C-term Lys<br>Deamidation (NQ)<br>Oxidation (MW)<br>Glycation (K)<br>Succinimide (ND)<br><br>**BSA**<br>Deamidation (NQ)<br>Oxidation (MW)<br>Phosphorylation (STY)<br>Glycation (K) |

## Data processing with Chromeleon CDS

The target peptide workbook created within BioPharma Finder software was imported into Chromeleon CDS version 7.3, and the data were analyzed using the ICIS MS peak detection algorithm. Table 6 summarizes parameter settings applied for PQA quantitation.

**Table 6. Chromeleon CDS parameter settings for target PQA monitoring and quantitation**

| MS chromatograms | Setting |
|---|---|
| **Mass precision** | 5 decimal places |
| **Mass tolerance (manually defined)** | 5 ppm |
| **Smoothing** | None |
| **Composite scoring** | **Setting** |
| **Pass score if at least** | 2 criteria passed |
| **Fail score if less than** | 1 criteria passed |
| **General MS criteria** | **Setting** |
| **Isotopic dot product** | ≥ 0.9000 |
| **Mass accuracy** | ≤ 5.00 ppm |
| **Peak apex alignment** | ≤ 0.50 min |

## Results and discussion

To compare the performance of the three UHPLC systems of the Vanquish platform, BSA and NISTmAb tryptic digests were analyzed using gradients of different lengths (Figure 2). A first data set was obtained analyzing ten replicate injections of the two samples on each instrument. A second data set was acquired two weeks later to perform intermediate precision evaluation on each platform. Instrument methods for both analyses were transferred across platforms using the automatic method translation available on Chromeleon CDS, including the translation from the Vanquish Flex UHPLC to the Vanquish Tandem LC system for Tandem LC-MS analysis (Figure 3).

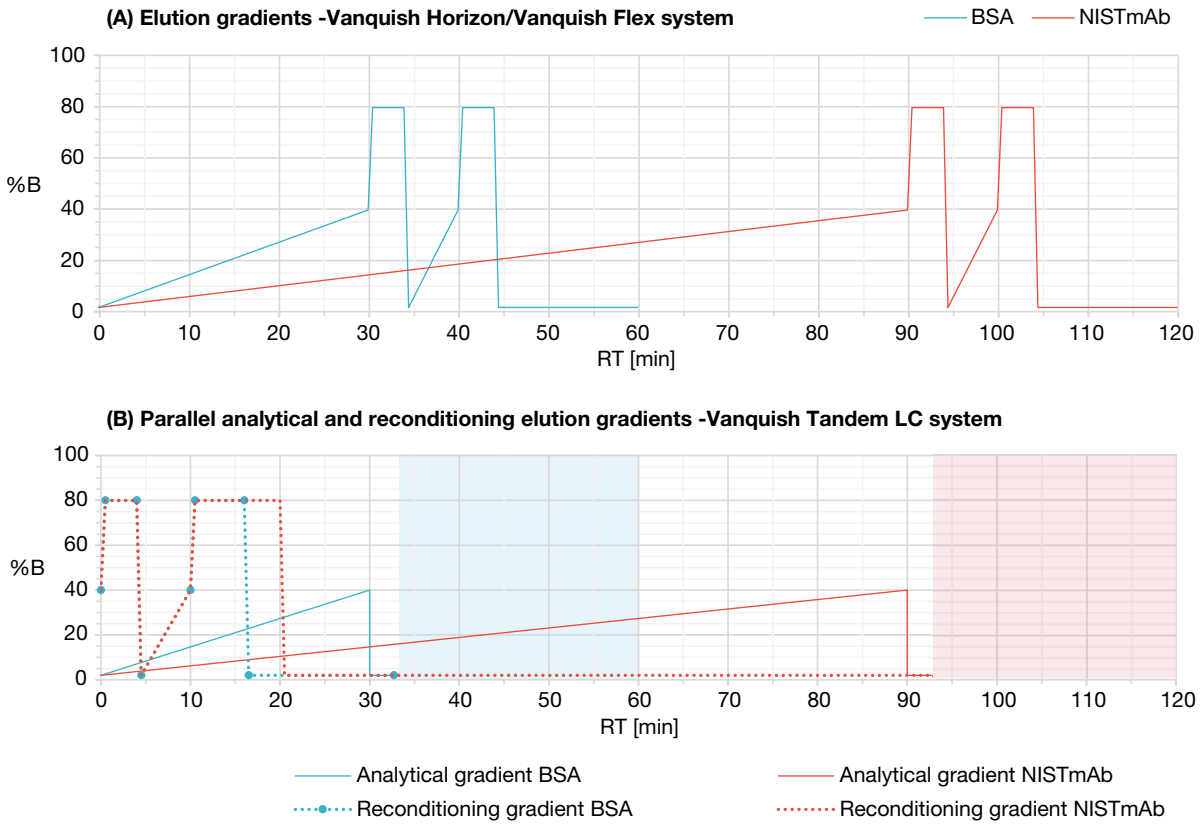



**Figure 2. Summary of elution gradients applied for BSA and NISTmAb samples.** (A) 30 min gradient with 60 min total run time for BSA digest (blue) and 90 min gradient with 120 min run time for NISTmAb digest (red), applied on both the Vanquish Horizon and Vanquish Flex systems. (B) 30 min analytical gradient on column 1 (blue solid line) with parallel reconditioning gradient using a step gradient on column 2 (blue dotted line) applied for BSA digest, and 90 min analytical gradient on column 1 (red solid line) with parallel reconditioning gradient applied on column 2 (red dotted line), applied for NISTmAb digest on the Vanquish Tandem LC system. The blue and red shaded areas indicate the time savings achieved by applying the Tandem operation of two columns with dedicated analytical and reconditioning pumps.

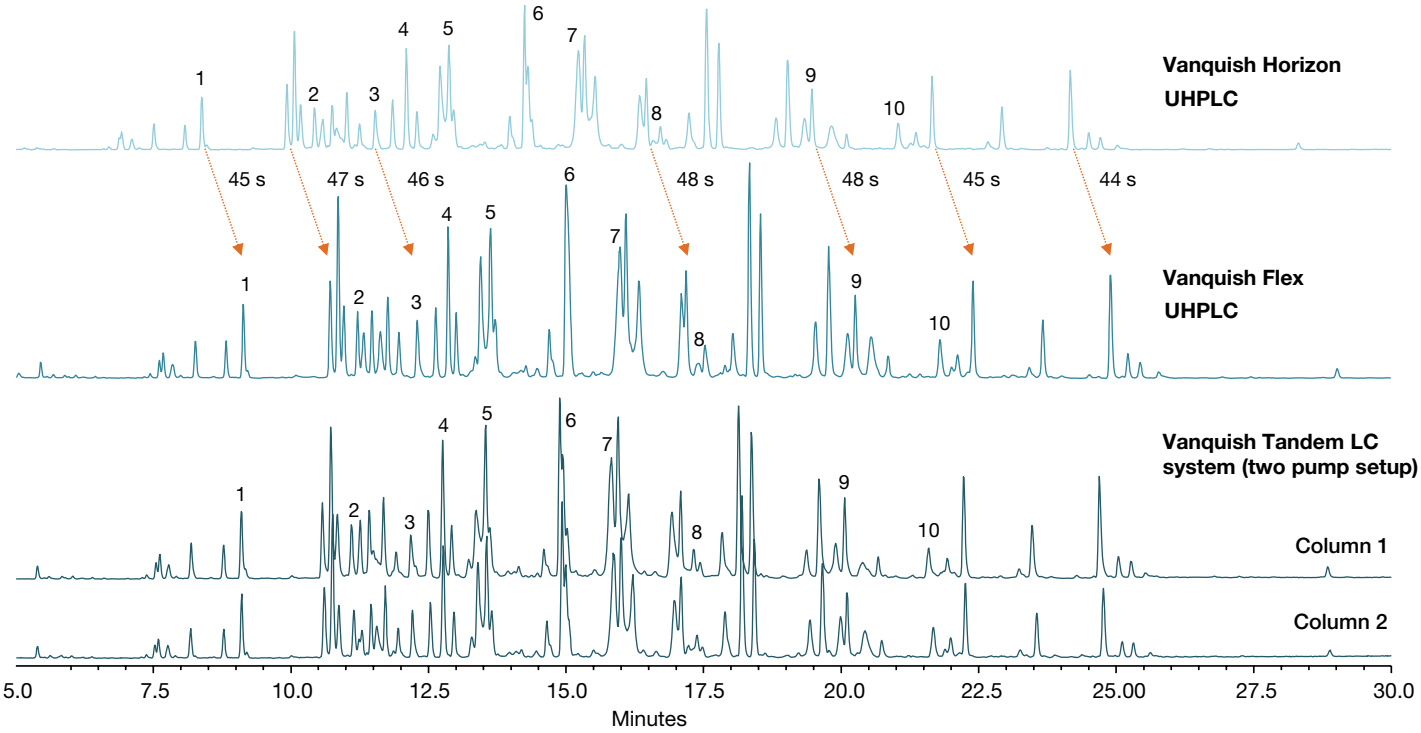



**Figure 3. Total ion chromatograms obtained from BSA digest sample, separated via a 30 min gradient on each of the three UHPLC systems.** The orange arrows indicate a constant shift across the entire chromatogram of ~46 s observed for the Vanquish Flex system.

Figure 3 presents the total ion chromatograms obtained on the three different systems and on both columns of the Vanquish Tandem LC instrument for the BSA digest sample. A distinct delay of the entire chromatogram of approximately 46 s was observed on the Vanquish Flex system compared to the Vanquish Horizon system. As for this sample a rather short and steep gradient was applied, the difference in the systems GDV of 175 µL for the Vanquish Horizon system compared to 340 µL for the Vanquish Flex system is the root cause for this small yet very reproducible difference in retention time.

Figure 4 represents the total ion chromatograms obtained from the analysis of NISTmAb digest applying a 90 min gradient.

Here, the chromatograms appear very well aligned. Only upon very close inspection of individual peak areas it is possible to observe that the GDV difference seems to have a greater impact in the first part of the chromatograms compared to peaks eluting later that are better aligning for the three instruments. As demonstrated in the expanded area around peak 2 at the earlier retention time 9.5–12.5 min, smaller shifts in retention time for the Vanquish Flex system compared to the Vanquish Horizon of ~14 s can be observed while peak shapes in regard to height and width are not affected. However, the observed small RT shifts only apply to the earlier part of the chromatogram. As the second expanded region around peak 8 at RT 42.0–44.0 min demonstrates, no further delays in RTs are observed.

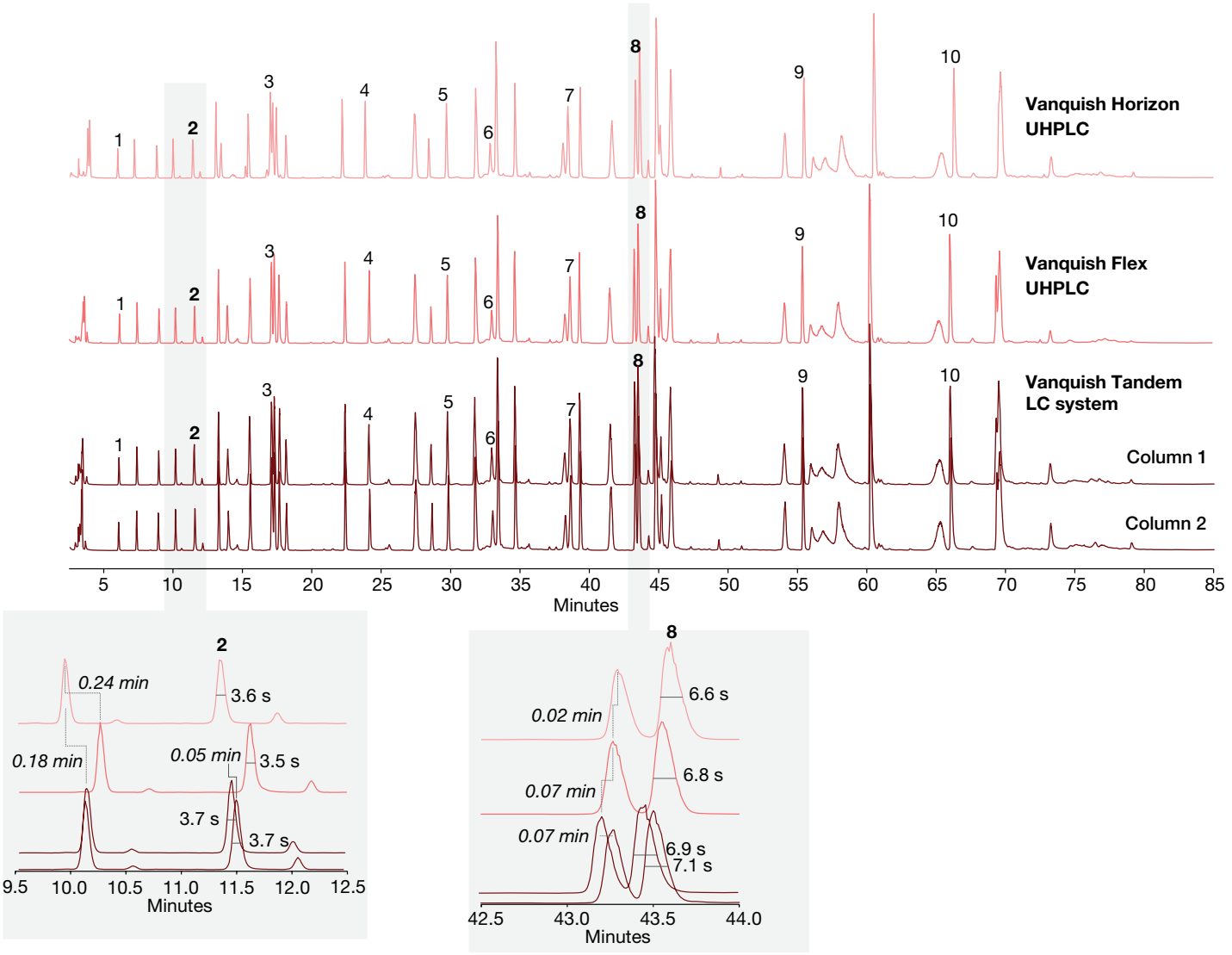



**Figure 4. Total ion chromatograms obtained from NISTmAb digest sample, separated via a 90 min gradient on each of the three UHPLC systems.** The expanded regions of the chromatograms between 9.50 and 12.50 min (left) and 42.5 and 44 min (right) highlight that the peak width is preserved on the three platforms compared in the study as indicated by the horizontal lines, whereas RT shifts are indicated by the vertical lines.

In-depth data comparison across the three selected instruments of the Vanquish platform was performed on the basis of several parameters (Table 7) including reproducibility of the peak area (peak area %CV ≤ 15), reproducibility of the retention time (RT %CV ≤ 2), full width at half maximum of the peak (FWHM %CV ≤ 7 s for BSA and 10 s for NISTmAb), mass accuracy (≤ 5 ppm), and reproducibility of post-translational modifications quantitation (%CV ≤ 10). The peptides selected for this study were chosen across the chromatographic profiles and are represented in Figure 5A and 5B for BSA and NISTmAb, respectively.

A summary of the data obtained for the NISTmAb sample is presented in Table 7. While excellent retention time reproducibility is shown on the two data sets for all three instruments, slightly different performances are observed on the peak area reproducibility with all the values still passing the evaluation criteria set for a CV% < 15. Peak width is another critical factor, and it was possible to observe good performances on all peaks included in the study, except for peak 9 that is consistently giving values of around 12 s, which is likely due to the intrinsic behavior of this peptide where the native form and its deamidated form elute very close in the conditions used for the analysis.

**Table 7. Evaluation of chromatographic performance for the NISTmAb peptide mapping analysis**

| | Vanquish Horizon UHPLC (n=20) | | | | | | Vanquish Flex Binary UHPLC (n=20) | | | | | | Vanquish Duo UHPLC (n=40) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **NISTmAb peptides** | Peak area (avg.) | %CV (area) (≤15%) | Avg. RT (min) | %CV (RT) (≤2%) | Avg. FWHM (s) (≤10) | %CV (FWHM) (≤15%) | Peak area (avg.) | %CV (area) (≤15%) | Avg. RT (min) | %CV (RT) (≤2%) | Avg. FWHM (s) (≤10) | %CV (FWHM) (≤15%) | Peak area (avg.) | %CV (area) (≤15%) | Avg. RT (min) | %CV (RT) (≤2%) | Avg. FWHM (s) (≤10) | %CV (FWHM) (≤15%) |
| **1.** EEMTK | 1.78E+09 | 10.6% | 5.90 | 1.5% | 3.46 | 2.6% | 2.08E+09 | 7.6% | 6.24 | 1.2% | 3.64 | 2.4% | 1.89E+09 | 5.6% | 6.05 | 1.0% | 3.79 | 2.6% |
| **2.** VEIK | 3.30E+09 | 6.4% | 11.29 | 1.2% | 4.59 | 2.2% | 3.63E+09 | 6.2% | 11.74 | 1.1% | 4.60 | 3.4% | 3.40E+09 | 2.9% | 11.52 | 0.8% | 4.72 | 2.5% |
| **3.** NQVVLK | 9.36E+09 | 8.7% | 16.87 | 1.0% | 5.78 | 3.1% | 1.03E+10 | 6.3% | 17.31 | 0.9% | 5.72 | 3.3% | 9.57E+09 | 3.1% | 17.08 | 0.7% | 5.93 | 3.8% |
| **4.** VDNALQSGNSQESVTEQDSK | 6.11E+09 | 12.4% | 23.73 | 0.5% | 4.69 | 1.9% | 6.72E+09 | 8.7% | 24.25 | 0.3% | 4.78 | 3.4% | 5.82E+09 | 9.6% | 24.13 | 0.3% | 4.88 | 4.0% |
| **5.** DTLMISR | 8.45E+09 | 10.6% | 29.55 | 0.7% | 5.83 | 2.8% | 9.17E+09 | 6.9% | 29.98 | 0.4% | 5.84 | 2.6% | 8.69E+09 | 3.4% | 29.79 | 0.4% | 5.92 | 3.2% |
| **6.** SLSLSPG | 3.70E+09 | 9.8% | 32.71 | 0.5% | 7.59 | 1.9% | 4.08E+09 | 6.2% | 33.15 | 0.4% | 7.61 | 2.2% | 3.78E+09 | 2.4% | 32.98 | 0.3% | 7.72 | 2.7% |
| **7.** DIQMTQSPSTLSASVGDR | 9.98E+09 | 12.5% | 38.33 | 0.4% | 8.63 | 2.7% | 1.07E+10 | 7.8% | 38.75 | 0.2% | 8.46 | 3.7% | 9.93E+09 | 4.1% | 38.62 | 0.2% | 8.58 | 3.8% |
| **8.** FNWYVDGVEVHNAK | 3.07E+09 | 13.3% | 43.46 | 0.6% | 6.22 | 5.2% | 3.04E+09 | 11.9% | 43.77 | 0.4% | 6.23 | 5.2% | 2.75E+09 | 7.7% | 43.58 | 0.4% | 6.42 | 7.2% |
| **9.** GFYPSDIAVEWESNGQPENNYK | 7.91E+09 | 15.0% | 53.94 | 0.4% | 11.69 | 2.8% | 8.56E+09 | 9.7% | 54.26 | 0.2% | 11.83 | 2.1% | 7.77E+09 | 4.8% | 54.12 | 0.3% | 12.00 | 3.5% |
| **10.** ALEWLADIWWDDKK | 1.77E+10 | 12.4% | 66.07 | 0.5% | 7.96 | 6.7% | 1.83E+10 | 9.2% | 66.26 | 0.3% | 7.50 | 4.7% | 1.76E+10 | 4.9% | 66.11 | 0.3% | 8.10 | 8.2% |
| Pass or Fail | | Pass | | Pass | Fail | Pass | | Pass | | Pass | Fail | Pass | | Pass | | Pass | Fail | Pass |

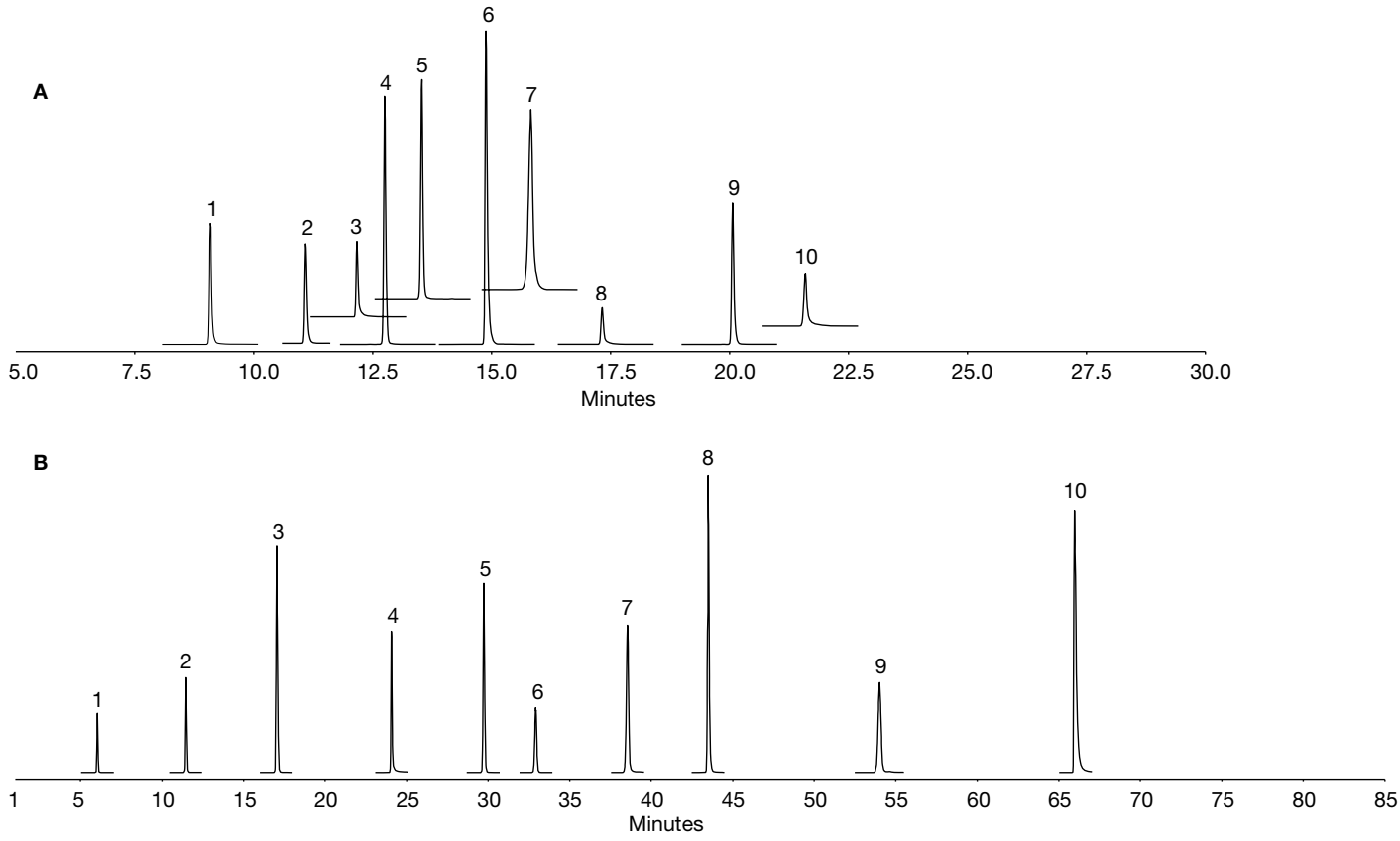



**Figure 5. Extracted ion chromatograms for the 10 peptides in the BSA sample (A) and the 10 peptides in the NISTmAb sample (B) selected for in-depth analysis**

**Table 8. Summary of PTMs evaluation on BSA (top) and NISTmAb (bottom).** Intermediate precision data are based on two data sets performed two weeks apart (n=20 for the Vanquish Flex system and Vanquish Horizon system and n=40 for the Vanquish Tandem LC system).

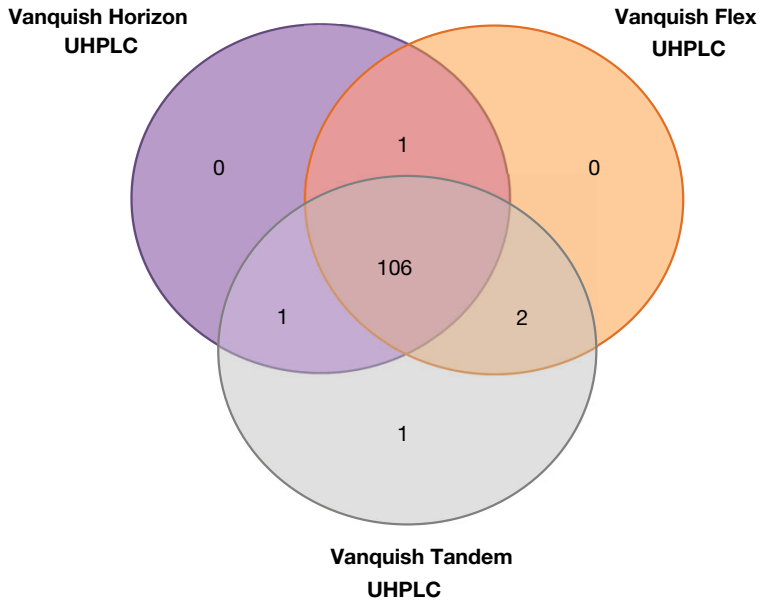
| | | Intermediate precision | | | | | | Cross-platform precision | |
|---|---|---|---|---|---|---|---|---|---|
| | | Vanquish Horizon UHPLC (n=20) | | Vanquish Flex UHPLC (n=20) | | Vanquish Tandem UHPLC (n=40) | | Vanquish Horizon/Flex/Tandem (n=80) | |
| BSA peptide | Description | %Mod. (Avg.) | %CV | %Mod. (Avg.) | %CV | %Mod. (Avg.) | %CV | %Mod. (Avg.) | %CV |
| HLVDEPQ$^{408}$N$^{409}$LIK | Deamidation | 2.41 | 6.68% | 2.35 | 7.85% | 2.32 | 6.92% | 2.35 | 7.15% |
| N$^{123}$ECFLSHK | Deamidation | 3.94 | 2.44% | 3.82 | 2.00% | 3.84 | 1.79% | 3.86 | 2.32% |
| K$^{548}$QTALVELLK | Glycation | 26.65 | 1.70% | 26.53 | 2.48% | 26.80 | 1.44% | 26.69 | 1.83% |
| KQ$^{549}$TALVELLK | Deamidation | 0.66 | 4.70% | 0.68 | 7.12% | 0.67 | 3.79% | 0.67 | 5.19% |
| NISTmAb peptide | Description | %Mod. (Avg.) | %CV | %Mod. (Avg.) | %CV | %Mod. (Avg.) | %CV | %Mod. (Avg.) | %CV |
| GFYPSDIAVEWESN$^{387}$GQPENNYK | Succinimide | 0.60 | 3.70% | 0.58 | 4.50% | 0.58 | 3.40% | 0.59 | 1.97% |
| DTLM$^{255}$ISR | Oxidation | 2.09 | 10.10% | 2.29 | 16.40% | 2.09 | 9.00% | 2.16 | 5.35% |
| EEQYN$^{300}$STYR | A2G0F | 39.36 | 1.78% | 37.87 | 2.58% | 36.20 | 3.90% | 37.41 | 4.70% |
| EEQYN$^{300}$STYR | A2G1F | 37.67 | 1.80% | 39.26 | 2.27% | 39.95 | 1.31% | 39.21 | 2.92% |
| EEQYN$^{300}$STYR | A2G2F | 8.49 | 1.22% | 8.61 | 3.58% | 9.38 | 3.83% | 8.97 | 5.76% |
| EEQYN$^{300}$STYR | A1G0F | 2.78 | 19.97% | 2.86 | 5.70% | 2.92 | 7.85% | 2.87 | 11.52% |
| EEQYN$^{300}$STYR | A1G1F | 3.30 | 4.43% | 3.23 | 2.30% | 3.16 | 2.72% | 3.21 | 3.65% |
| EEQYN$^{300}$STYR | A1G1M4F/A1G0M5F | 1.03 | 4.97% | 1.15 | 5.57% | 1.09 | 5.08% | 1.09 | 6.45% |
| EEQYN$^{300}$STYR | A2Ga1G1F | 1.55 | 2.89% | 1.50 | 4.53% | 1.43 | 4.92% | 1.48 | 5.61% |
| EEQYN$^{300}$STYR | A2Ga2F | 0.68 | 4.83% | 0.64 | 5.09% | 0.60 | 3.77% | 0.63 | 6.63% |
| EEQYN$^{300}$STYR | M5 | 1.02 | 7.81% | 0.92 | 16.28% | 0.73 | 6.86% | 0.85 | 18.30% |
| EEQYN$^{300}$STYR | Unglycosylated | 0.79 | 4.56% | 0.74 | 4.68% | 0.70 | 3.10% | 0.73 | 6.36% |

For a successful peptide mapping analysis, reproducibility and consistency of the results obtained after data processing is critical. To prove comparable results can be obtained for the three instruments, a single LC-MS/MS analysis on each platform was analyzed using BioPharma Finder 4.1 software. The results are summarized in Figure 6. Sequence coverage shows identical figures for all instrument configurations. Filters were applied to the component list obtained from the data processing, excluding adducts, nonspecific, gas phase ions, and unknowns and considering only components with delta ppm within ±5 ppm, confidence score ≥ 95 and including up to 1 missed cleavage. The list of peptides obtained from each instrument were compared using a Venn diagram (Figure 6), showing excellent overlap of the 95.5% of the components.

As the key data obtained after peptide mapping analysis rely on the quantitation of post-translation modifications (PTMs), it is critical to obtain reproducible results after methods are transferred to a different platform. For this reason, PTM levels were evaluated for the two data sets (n=80) using a targeted approach through Chromeleon CDS (Figure 7, Table 8). PTMs evaluated for both BSA and NISTmAb were chosen to include deamidation, oxidation, glycation, succinimide formation, and glycosylation, looking also at low abundant species with relative abundance down to 0.5%. Excellent reproducibility was observed in most cases for intermediate precision on the single platform, especially when using the Vanquish Tandem LC system, as demonstrated previously, and reproducible quantitation of the selected PTMs is seen across the platforms. The results obtained in this study demonstrate the suitability of all the platforms for peptide mapping analysis and to be included in a MAM workflow, with seamless method transfer and without affecting workflow results.

As demonstrated in Figures 3 and 4, the alignment of absolute retention times with the systems used in this comparative study and in their standard configurations is strongly dependent on the gradient duration and depth. Systems with larger gradient delay volumes, such as the Vanquish Flex Binary UHPLC, have longer times between injections and mobile phase changes, resulting in later eluting peaks in gradient separations, as it was observed in the case of BSA. Moreover, the elution pattern can be modified due to the longer isocratic holdup at the beginning.

However, there are two possible approaches to modify the systems to compensate for the GDV difference between the chromatographic systems. Firstly, the applied method can be adapted by implementing an isocratic hold to compensate for the difference in GDV. The extent and the real need depend on the exact gradient as the data presented here underline. Alternatively, hardware modifications can be implemented by changing the mixer and the sample loop, e.g., the same 35 µL mixer used in the Vanquish Horizon system could be built into a Vanquish Flex Binary system resulting in matching GDVs.




| NISTmAb heavy chain | Sequence coverage % |
|---|---|
| Vanquish Horizon | 100.00 |
| Vanquish Flex | 100.00 |
| Vanquish Tandem – column 1 | 100.00 |
| Vanquish Tandem – column 2 | 100.00 |
| **NISTmAb light chain** | **Sequence coverage %** |
| Vanquish Horizon | 98.59 |
| Vanquish Flex | 98.59 |
| Vanquish Tandem – column 1 | 98.59 |
| Vanquish Tandem – column 2 | 98.59 |

**Figure 6. Summary of the LC-MS/MS analysis of the NISTmAb peptide mapping obtained on the three systems, based on a single run each.** The Venn diagram demonstrates 106 of the 111 components were detected on all three systems while keeping sequence coverage results identical for all the analyses.

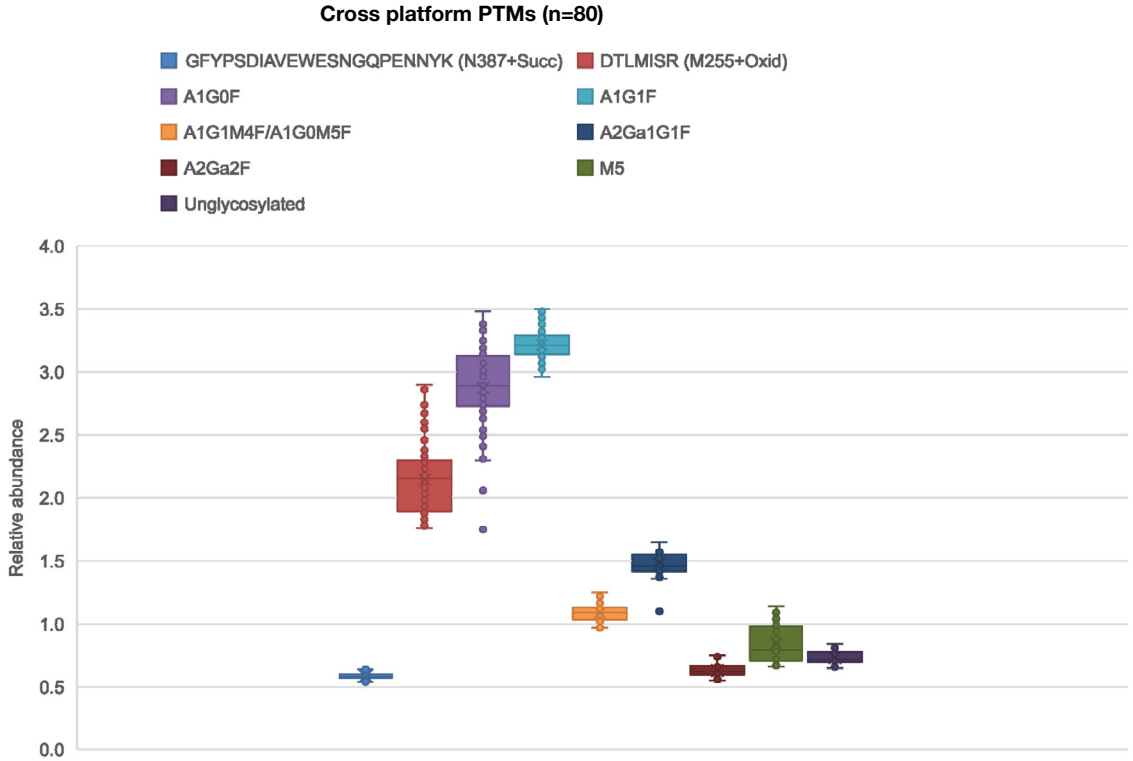



**Figure 7. Box plots of selected post-translation modifications for peptides from NISTmAb digest representing all data obtained on the replicate experimental data sets from all three systems (n=80)**

## Conclusions

- The three Vanquish UHPLC systems used in this study showed excellent retention time precision for the shorter runs using BSA as well as the longer gradient times using NISTmAb digest.
- Assessment of transferability of methods across systems showed that for shorter gradient length, a consistent chromatographic elution profile was obtained with a constant shift throughout the entire chromatogram. For longer gradient lengths, only a small yet negligible shift was observed in the first part of the gradient.
- Assessment of quantitative results obtained from both samples on all three systems showed excellent consistency of intermediate and cross system precision, even for the modified peptides with an abundance <5%.
- The Vanquish Tandem LC system demonstrated the benefit of increased throughput by 23% and 46%, respectively, compared to a 120 min and 60 min total run time (elution and reconditioning gradient) and provided highly consistent and reproducible results from the two columns used in Tandem mode.

## References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