Nano and Microscale Peptide Mapping by LC-UV-Orbitrap-Astral Detection of Monoclonal Antibodies with Fraction Collection for Detailed Analysis

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Abstract

Purpose: Demonstrate nano- and micro-chromatography with fraction collection of tryptic digests of monoclonal antibodies with combined UV and Astral data collection.

Methods: DDA and DIA methods were used on a Thermo Scientific™ Orbitrap™ Astral™ Mass Spectrometer online with nano- and micro-UV detection and fraction collection.

Results: Limits of detection were defined to identify by UV the smallest peak required for full sequence identification. Using microflow with a 1 mm ID column, 70 pmols is required while 80 fmols is required for nanoscale chromatography. Fraction collection of ~15-22 µL per vial was obtainable in microflow mode.

Introduction

UV detection of tryptic peptide maps (multi-attribute method, MAM or critical quality attributes, CQA) is often the major technique in verification of the primary structure, quantitation, and tracking of co- and post-translational modifications (PTMs) or product quality attributes in protein-based biopharmaceutical drugs in a QA/QC environment. This technique is gradually being supplemented by high resolution mass spectrometry, traditionally found in research and development laboratories. Smaller biopharma companies working with orphan drugs or first to market candidates may not have access to large biomanufacturing for early-stage clinical trials and have a need for nano or micro chromatography sensitivity limits (operating in the nanogram to microgram rather than microgram to milligram levels). The ability to transition effortlessly from the UV to mass spectrometry either online or offline with fraction collection is beneficial.

Materials and methods

Sample Preparation

Trastuzumab (Herceptin®, MedChemExpress) was digested using Thermo Scientific™ SMART Digest™ Trypsin (heat stabilized beads) in a two-step method[1] to reduce process induced modifications. One milligram of protein was digested with 15 µL of trypsin beads with the addition of 5 mM Thermo Scientific™ Bond-Breaker™ TCEP to the SMART Buffer at 70 ºC for 15 minutes with shaking. The solution was centrifuged, and the supernatant was transferred to another tube of beads for an additional 30-minute digestion at 37 ºC. The beads were centrifuged, and the solution was filtered using a Thermo Scientific Titan3™ Nylon Syringe Filter (0.2 µm x 4 mm).

Instrument Methods

The samples were run online with UV detection (3 nL flow cell) with fraction collection using the Thermo Scientific™ Vanquish™ Neo UHPLC System with the Vanquish variable wavelength detector F and the Vanquish integrated fraction collector. The mass spectrometry was shown using traditional data dependent analysis (DDA) or using data independent analysis (DIA) on an Astral detector. Nano chromatography was performed using direct injection onto either a Thermo Scientific™ Double nanoViper™ PepMap™ Neo UHPLC 75 µm x 15 cm column (DNV75150PN, 350 nL/min, 5-30% ACN / 0.1% FA in 45 minutes) or a 5.5 cm High Throughput µPAC™ Neo HPLC column (COL-CAPHTNEOB, 1.25 µL/min, 1-45% ACN / 0.1% FA in 45 minutes); both columns were run at 50 ºC using a 10 µm EASY-Spray™ ES993 Nano emitter. Microflow chromatography was performed using a Thermo Scientific™ Acclaim™ PepMap™ 100 C_{18} 1 mm x 15 cm HPLC column (164711, 50 µL/min, 1-30% ACN / 0.1% FA in 53 minutes at 35 ºC). All peaks were fraction collected, weighed, and analyzed in triplicate for collection accuracy and reproducibility. The fraction collector delay was calculated theoretically and then verified by UV absorption using a Thermo Scientific™ NanoDrop™ One across an LC peak collection looking for the highest absorption at 214 nm.

Data Analysis

Mass data was analyzed for coverage using Thermo Scientific™ Biopharma Finder™ 5.2 software and imported into Thermo Scientific™ Chromeleon™ 7.3.1 or Thermo Scientific™ TraceFinder™ 5.1 software for quantitative analysis and tracking.

Results

Microflow LC-UV-MS-FC

Microflow chromatography allows for the direct coupling of the UV detector to either the mass spectrometer or the integral fraction collector with 50 µm nanoViper tubing. Seventy pmols or 10.5 µg was loaded on column.

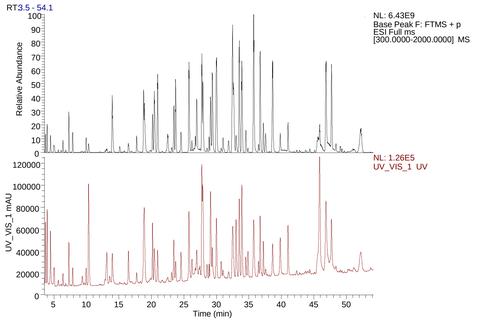


Figure 1. The top pane contains the base peak plot of 70 pmols of digest in full scan mode utilizing Orbitrap detection. The bottom pane shows the UV detection of the sample. This amount of loading material was determined by a Biopharma Finder search of the data; the smallest chromatographic peak from UV data that produces full coverage of the heavy and light chains was mapped to the corresponding peak in the mass spectral data. See Figure 3 for a further explanation.

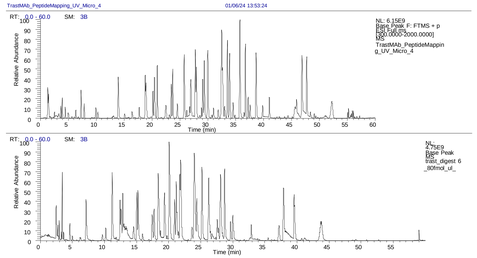
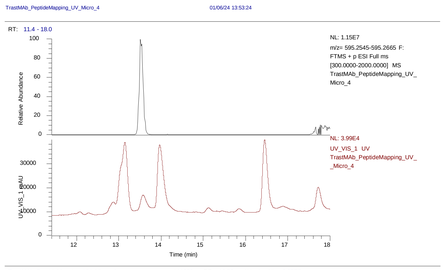


Figure 2. Comparison of 70 pmols in micro mode to 80 fmols (12 ng) in nano mode.



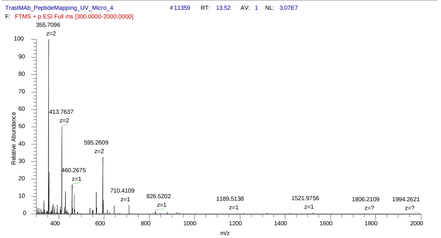


Figure 3. EEQYNSTYYR from the heavy chain of Trastuzumab is the peptide with the poorest UV response which is required to map the full sequence of the antibody. Appearing at 13.52 minutes in the microflow chromatogram, the sequence represents 0.17% of the tallest assignable mass spectral peak. The top chromatogram of the top pane shows the extracted ion chromatogram of the peak from the full scan Orbi data at 120,000 resolution. The lower chromatogram shows the UV data (3 nL fused silica flow cell) for the same peak. The bottom pane shows the spectra in the Orbi at the peak apex.

Fraction Collection of Peptides in Micro Flow Mode

The integrated fraction collector was plumbed using 50 um Viper™ tubing. There is currently no support for the Neo Pump in the fraction collection software, hence the automated delay volume determination could not be used. The delay volume was estimated at 4.5 µL. Fractions were time collected across the 722.2 $^{+2}$ standard peak of BSA digest. The collected fractions were analyzed on a NanoDrop UV to find the most intense for time alignment; the proper delay volume was 4.7 µL. The actual MAb sample was fraction collected in 'Collect by peak' mode using the UV signal. The first injection was imported into the fraction collection tab of Chromeleon and peak detection parameters were adjusted to collect all real peptides of the digest. The collection regions are shown in Figure 6.

Comparing DDA to DIA on the Astral

Nanoflow chromatography was used to separate the tryptic digest using a 5.5 cm High Throughput µPAC™ Neo HPLC column (COL-CAPHTNEOB, 1.25 µL/min). The 'radially elongated pillars', of this configuration, has the longer side of the rectangular pillar oriented radially, while the shorter side aligns axially.[2] The design of radially elongated pillars results in a more meandering flow path, allowing for additional separation length to be achieved within a constrained space. As the ratio of the radial direction increases relative to the axial direction, the column's separation approaches that of an open tubular column. The eluting peptides were sprayed using a 10 um ES993 EASY-Spray emitter.

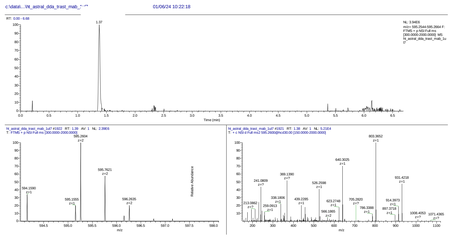


Figure 4. The top pane shows the extracted ion chromatogram of the EEQYNSTYYR peptide from the heavy chain of Trastuzumab. The lower left pane shows the mass spectrum of the peak at the apex. The lower right pane shows a traditional DDA scan acquired on the Astral (data shown in Qual Browser which doesn't list the Astral detector in the filter). Quadrupole isolation at a window of 1 m/z was used with higher-energy collisional dissociation at 30 NCE. The AGC was set to 300%. Relative or absolute quantification could be performed using the Orbi full scan data.

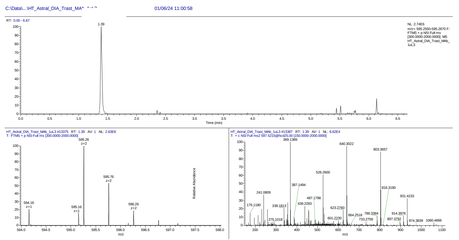


Figure 5. The top pane and lower left pane show the same data as Figure 4. The lower right pane shows the DIA ms/ms spectra that contains the eluting peptide's fragment ions. The same b and y ions are observed with slightly different ion ratios because of the mass centering of the window. Quantification could be performed using either the full scan Orbi data or the PRM of the appropriate b and y ions from the Astral Data.

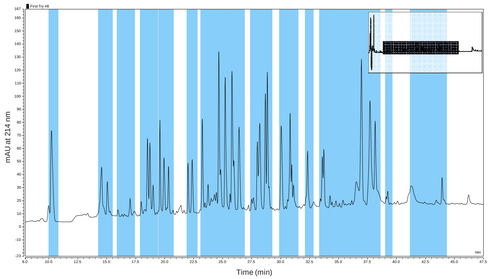


Figure 6. This is the peaks collected in micro mode on the fraction collector. Fractions in blue were collected into a 54 vial rack with 300 µL ultra high recovery vials. The peaks were collected in triplicate, weighed, and reinjected for the determination of the qualitative accuracy. The average RSD of fractions by weight was 5.9%.

Conclusions

- Integration of UV and fraction collection with the Vanquish Neo in micro and nano mode
- DIA and DDA can both be used effectively for QA/QC peptide mapping
- Nanogram and microgram amounts of MAbs can produce fruitful QA/QC data.

References

1. *Analytical Chemistry* 2022, 94, 17195-17204
2. *Analytical Chemistry* 2015, 87, 7382-7388

Acknowledgements

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