APPLICATION NOTE

# Microchip CE/MS Peptide Mapping Workflow with Trypsin Digestion for the Analysis of Critical Quality Attributes of Monoclonal Antibodies


## Authors

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## Keywords

Peptide mapping, sample preparation, multi-attribute method (MAM), capillary electrophoresis, ZipChip, CE-MS, monoclonal antibody


## Highlights

- Microchip based Capillary Electrophoresis (CE) is coupled with high resolution accurate mass (HRAM) measurement of tryptic peptides for the analysis of monoclonal antibodies
- An optimized ZipChip CE/MS compatible NIST mAb digestion workflow for peptide mapping (Figure 1)
- CE-MS analysis enables high sequence coverage and correct monitoring of critical quality attributes (CQAs) including deamidation, succinimide formation, oxidation, and N-glycosylation

## Introduction

Peptide mapping is considered the gold standard for the analysis of biopharmaceuticals, including monoclonal antibodies. The main advantage of this bottom-up technique is that the protein is analyzed at the peptide level, revealing important information about the primary sequence and the location of each post-translational modification (PTM) that might be present. Peptide mapping analysis is usually performed through long workflows involving both extensive sample handling and long instrument analysis times, typically by LC-MS. Total analysis times for such workflows can reach 2 days for a single sample, preventing high-throughput and quick turnaround, which are highly desirable in fast paced R&D environments.


Microchip CE-MS technology applied to peptide mapping analysis has been previously explored by Dykstra *et al.*[1], demonstrating the advantage of short analysis times and unique separation mechanisms that allows separation of modifications such as isoaspartic acid (isoAsp) variants.

| | |
|---|---|
| Sample | • 100 µg IgG to 1 mg/mL Solution<br>• Diluent: Guanidine HCl (7 M) + Tris (100 mM), pH 8.3 |
| Reduction RT, 30 min | • Add 2 µL DTT Solution (final DTT conc. 10 mM)<br>• DTT Solution: 500 mM in Diluent |
| Alkylation | • Add 4 µL IAC Solution (final IAC conc. 20 mM)<br>• IAC Solution: 500 mM in Diluent<br>• Add 4 µL DTT Solution to terminate Alkylation |
| Buffer Exchange | • Bio-Spin P-6 Gel Column<br>• 100 mM Ammonium Acetate<br>• Purpose: Remove salt and reagents |
| Digestion 37°C, 30 min | • Add 10 µL of 1 µg/µL Trypsin Solution (1:10 ratio)<br>• Add 10% Formic Acids to terminate digestion<br>• 1:1 dilution with Acetonitrile |
| Sample ready for ZipChip/MS analysis | |

*Figure 1. NIST mAb Digest Workflow Optimized for Peptide Mapping by ZipChip CE/MS*

However, the protocol used in the paper involved additional steps for the sample preparation to eliminate sample buffer components such as Tris prior to analysis, which could potentially result in loss of peptides.

In this study, we developed an optimized protocol for sample preparation that is directly compatible with ZipChip CE-MS analysis and demonstrated equivalence of this protocol to a traditional LC-MS based peptide mapping approach. The analysis was performed using the ZipChip CE interface coupled to a Thermo Scientific Orbitrap Exploris 240 Mass Spectrometer. The entire peptide mapping analysis workflow including sample preparation, analysis and data processing was completed in less than two hours returning 99% sequence coverage and accurate determination of selected critical quality attributes (CQAs). This microchip CE-MS based workflow proves to be an attractive approach for high-throughput analysis for evaluation of monoclonal antibodies and for implementation into a Multi-Attribute Method (MAM) workflow.

## Materials and Methods

**Reagents and ZipChip consumables:** 8.0 M Guanidine Hydrochloride Solution, dithiothreitol (DTT) BioXtra, Iodoacetic acid (IAC) BioXtra, sodium hydroxide concentrate, Ammonium acetate (99.999% trace metal grade), trifluoroacetic acid (TFA) were all obtained from Sigma-Merck, UltraPure 1M Tris-HCl pH 7.5 (Invitrogen), Pierce™ Trypsin Protease, MS grade (Thermo Scientific™), Bio-Spin® P-6 Gel Columns, Tris Buffer (Bio-Rad), ZipChip Peptide Kit (908 Devices, Part No. 850-00034), ZipChip HR chip (908 Devices, Part No. 810-00194 ).

**Sample:** NISTmAb Humanized IgG1$_K$ Monoclonal Antibody Lot 14HB-D-002 (NIST, RM 8671) was obtained from Sigma-Merck.

**Instruments:** ZipChip-Ti interface was used on a Orbitrap Exploris 240 Mass Spectrometer (Thermo Scientific). Samples were delivered using a ZipChip autosampler.

**Data acquisition and processing:** All data were acquired through Chromeleon CDS 7.3.1 and processed through BioPharma Finder v5.1.

### ZipChip CE-MS compatible tryptic digest for monoclonal antibodies

In peptide mapping analysis of proteins, the preparation of tryptic digest usually requires the presence of salts that are either for denaturing protein secondary structure, or for controlling the pH of the solution where reduction, alkylation and, most importantly, digestion occur. Widely used buffers include guanidine

### ZipChip Method:

| | |
|---|---|
| **Field strength:** | 500 V/cm |
| **BGE:** | Peptide |
| **Injection volume:** | 8 nL |
| **Pressure Assist Start Time:** | 0.5 min |
| **Analysis Time:** | 15 minutes |
| **Chip Type:** | HR |

### MS Method:

| | |
|---|---|
| **Sheath gas** | 2 |
| **Full MS** | |
| **Scan range (m/z)** | 200-2,000 |
| **Resolution** | 120,000 at m/z 200 |
| **Capillary temperature (°C)** | 200 |
| **Normalized AGC target (%)** | 300 |
| **Max IT (ms)** | 20 |
| **Microscans** | 1 |
| **RF lens (%)** | 50 |
| **MS/MS settings** | |
| **Resolution** | 15,000 |
| **Isolation Window (m/z)** | 4 |
| **Intensity Threshold** | 1.0e5 |
| **Charge States** | 1-6 |
| **Loop Count** | 5 |
| **Normalized AGC target (%)** | 10 |
| **Max IT (ms)** | 50 |
| **Dynamic exclusion (s)** | 4 |
| **Microscans** | 1 |
| **Normalized Collision Energy (%)** | 30 |

hydrochloride or Tris-based buffers, which are not compatible with ZipChip CE-MS analysis and require removal prior to analysis. To allow a streamlined sample preparation directly compatible with ZipChip CE-MS analysis, we adapted and optimized an existing protocol that is used for LC-MS based peptide mapping analysis in our lab. This protocol ensures high sample reproducibility and low artificially induced modifications[2], and is therefore considered suitable for implementation into a MAM workflow.

Reduction and alkylation of IgG1 samples was performed as follows[2]. Briefly, to a 10 µL aliquot of the sample with a concentration of 10 mg/mL, 90 µL solution (7 M Guanidine HCl, 100 mM Tris, pH 8.3) was added, followed by 2.0 µL of 500 mM DTT (final concentration 10 mM). Sample was incubated for 30 minutes at room temperature.

For alkylation of the free cysteines, 4.0 µL of 500 mM IAC was added to the previously reduced sample and mixed by pipette action to give a final concentration of 20 mM IAC. The solution was incubated in the absence of light for 20 minutes at room temperature. Alkylation was stopped by further addition of 4.0 µL of 50 mM DTT.

Fully reduced and alkylated sample was buffer exchanged into 100 mM ammonium acetate buffer pH7 to eliminate residual reagents that were used in the previous steps and remove buffer salts that are incompatible with ZipChip. Buffer exchange was performed using BioSpin-6 columns (prewashed and equilibrated with 100 mM ammonium acetate) and 110 µL of buffer exchanged sample was collected into a new tube, ready for trypsin digestion.

100 µL of LC-MS grade water was added to a vial of 100 µg trypsin to give a final concentration of 1 mg/mL. The solution was mixed by gentle vortexing. This solution was added in a 1:10 ratio to the buffer exchanged protein sample above. The resulting solution was incubated at 37 °C for 30 minutes. Digestion was halted by adding 10% formic acid (1:10 ratio in volume with the digestion solution) and the pH verified to be acidic (approximately pH 1.5). The final sample solution was mixed 1:1 (v/v) with acetonitrile to approximate the recommended peptide sample diluent conditions before transferring to low-binding chromatography glass vials with inserts.

Sample vials were placed in the autosampler sample rack held at 5 °C.

## Results and Discussions

### NIST mAb tryptic digest evaluation by LC-MS

The NIST tryptic digested sample was first analyzed by standard LC-MS, and also compared with a sample obtained using the standard tryptic digest protocol with Tris buffer. After the LC-MS analysis, the acquired raw data were processed using a non-targeted approach, which revealed no significant differences between the two samples. Upon a closer look using a targeted approach, it was possible to estimate the impact of the different sample preparation for the evaluation of NIST mAb PTMs, which resulted in a small decrease in the level observed for deamidation and succinimide formation. This was expected as the tryptic digestion was performed at a pH ~7, while more conventional buffers work at a pH around 8.

The sample was then analyzed using the ZipChip hyphenated with an Orbitrap Exploris 240 MS. For the delivery of the sample, the ZipChip default peptide method was modified to inject 8 nL of sample instead of 5.5 nL. This ensured more intense signals and detection of some low abundant peptides. The MS settings were slightly modified to suit the performance of the CE separation; in particular, dynamic exclusion of species from MS/MS fragmentation events was lowered to 4 seconds to balance the narrower peaks in the electropherogram.

Samples were analyzed in triplicate with a BGE refresh and transfer of a new aliquot of sample to maximize reproducibility (Figure 2). Data were analyzed using BioPharma Finder v.5.1 software to evaluate sequence coverage and PTMs abundance.

Data processing was performed by filtering the components to consider peptides with up to only one missed cleavage with the exception of TKPREEQYNSTYR, which was also included because

# A

Created on 12/01/22
Minimum MS Signal = 0
Data File = NIST_newDigest30min_Inj8ul_HR3387_rep1_52.raw
Protease = Trypsin

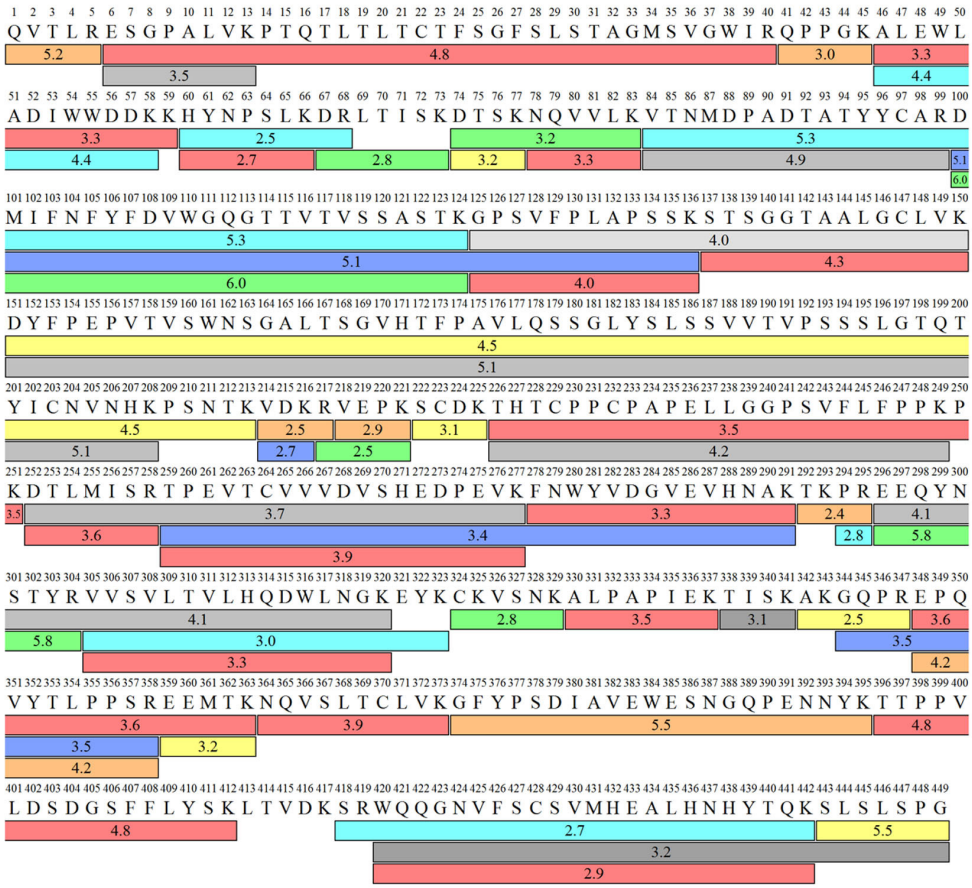
Minimum Recovery = 0%
Minimum Recovery of Overlapping Peptides = 0%
Minimum Confidence = 0
Maximum Mass = 7000

| Proteins | Number of MS Peaks | MS Peak Area | Sequence Coverage | Abundance (mol) |
|---|---|---|---|---|
| 1:NISTmAb HC | 154 | 66.8% | 98.9% | 59.70% |
| 2:NISTmAb LC | 67 | 33.2% | 98.1% | 40.30% |
| Unidentified | 0 | 0.0% | | |

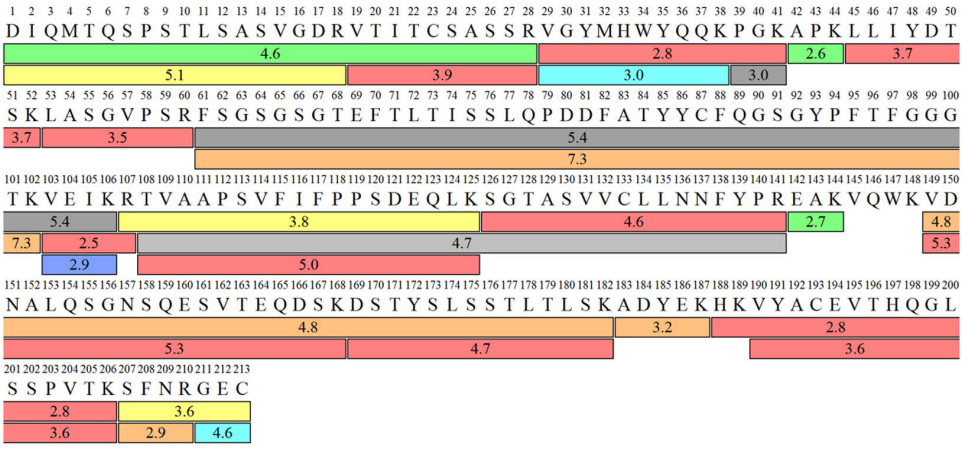
Color code for peptide recovery

| >50.0% | >20.0% | >10.0% | >5.0% | >2.0% | >1.0% | >0.5% | >0.2% | >0.1% | >0.0% |
|---|---|---|---|---|---|---|---|---|---|
| good | | | fair | | | poor | | | |

## NISTmAb HC




## NISTmAb LC



**B**

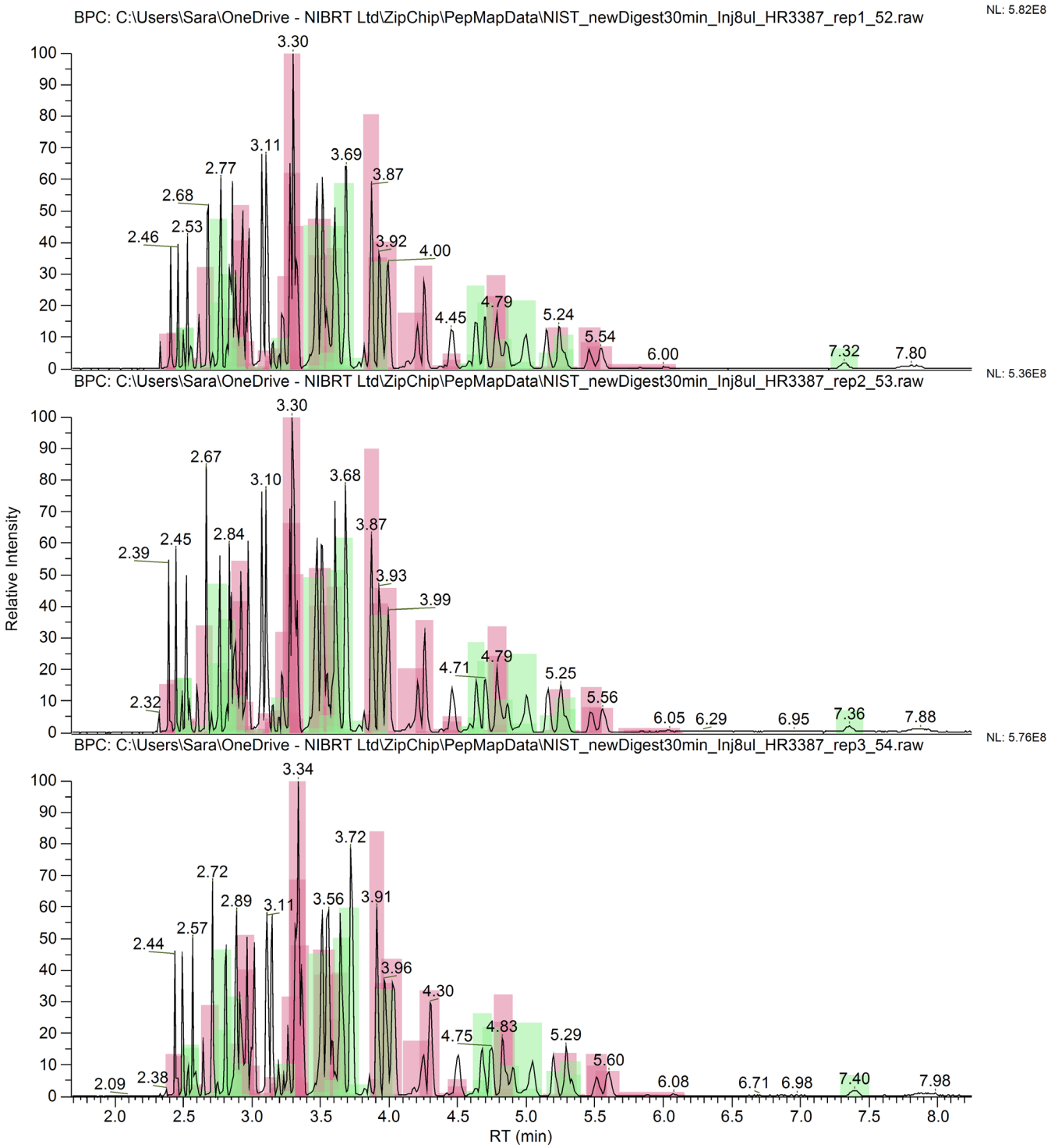



*Figure 2 – A) Sequence coverage map obtained after data processing of the CE-MS analysis of NIST mAb tryptic digest. B) Base peak electropherograms (BPE) of the three replicates peaks highlighted in red correspond to peptides of the heavy chain, while peaks highlighted in green correspond to the light chain.*

of its significant abundance for the evaluation of Fc N-glycan. Sequence was generated considering carboxymethylation as fixed modification for cysteine residues, pyro-glutamate as fixed modification of heavy chain N-termini glutamine and lysine clipping as fixed modification of heavy chain C-termini. Peptides containing sodium or potassium adducts, as well as those that correspond to nonspecific protease activity, gas phase generated ions, or unspecified modifications, were excluded. Identification was only accepted if mass accuracy fell within a ± 5 ppm range and with a confidence score ≥ 90. Good sequence coverage around 99% for both heavy and light chains was achieved for the three replicates.

Following sequence map coverage, data analysis was performed to evaluate the relative abundance of PTMs. Modifications evaluated in this study included oxidation, deamidation and succinimide formation as well as Fc N-glycan profiling (Table 1). The observed modifications were in line with previously published results[2] and their variability across replicates was minimal as indicated by low %RSD values, with only two modifications exceeding 10% RSD.

In particular, CE-MS analysis performed well in the evaluation of critical modifications such as M255 oxidation and PENNYK peptide variants (Figure 3). Deamidation is usually a challenging modification as its presence may result in minor chromatographic shift while the small mass shift (+1 Da) forces the use of high-resolution settings in the mass spectrometer to resolve[1].

| Chain | Residue + Modifications | Relative Abundance (%) | RSD (%) |
|---|---|---|---|
| HC | M255+Oxidation | 1.99 | 7.1 |
| HC | N300+A1G0 | 0.52 | 14.3 |
| HC | N300+A1G0F | 3.09 | 4.6 |
| HC | N300+A2G0F | 44.45 | 0.2 |
| HC | N300+A2G1F | 41.39 | 0.5 |
| HC | N300+A2G2F | 9.68 | 2.0 |
| HC | N300+A1G1F | 2.70 | 1.9 |
| HC | N300+Unglycosylated | 1.69 | 7.1 |
| HC | M361+Oxidation | 0.79 | 5.8 |
| HC | N387+Deamidation | 0.94 | 4.3 |
| HC | N387+Succinimide1 | 0.71 | 1.0 |
| HC | M431+Oxidation | 1.27 | 7.4 |
| LC | M4+Oxidation | 2.05 | 18.7 |

*Table 1 – PTMs relative abundance as estimated after analysis of CZE-MS data through BioPharma Finder 5.1 software.*

Deamidation is considered a CQA as it may lead to protein instability and change in the antigen binding properties when present in the variable regions. As a consequence, monitoring the abundance of this modification is pivotal for accurate evaluation of biotherapeutic stability, activity and safety. Efforts to accurately monitor this modification mainly result in longer experimental times to chromatographically resolve the modification from its unmodified form. By using CE-MS, it was possible to accurately identify and quantify this modification without extensive method optimization and using a total experimental time of 20 minutes, while obtaining almost baseline resolution of the two species. Examples of the resolution obtained for some modifications is showed in Figure 2A, zoom.

## Conclusions

Peptide mapping is a critical assay widely performed in the biopharma industry. Simplification of the analytical protocol and shorter analysis times are highly desiderable to allow quick turnaround and automation of sample preparation steps and analysis. In this study we demonstrated the suitability of a modified digestion workflow for peptide mapping analysis via CE separation. The protocol for sample preparation proposed here involves replacing Tris salt buffer for the trypsin digestion with ammonium acetate buffer. The workflow resulted in similar performance for CQA evaluation when compared to standard protocol but has the advantage of being directly compatible with ZipChip CE-MS analysis. NIST mAb peptide mapping was performed in less than 20 minutes per replicate, allowing excellent sequence coverage and accurate evaluation of the main PTMs. The described sample preparation workflow and short analysis times of ZipChip CE-MS make it a faster alternative to more standard LC based approaches and readily implemented in both academic and industrial characterization laboratories.

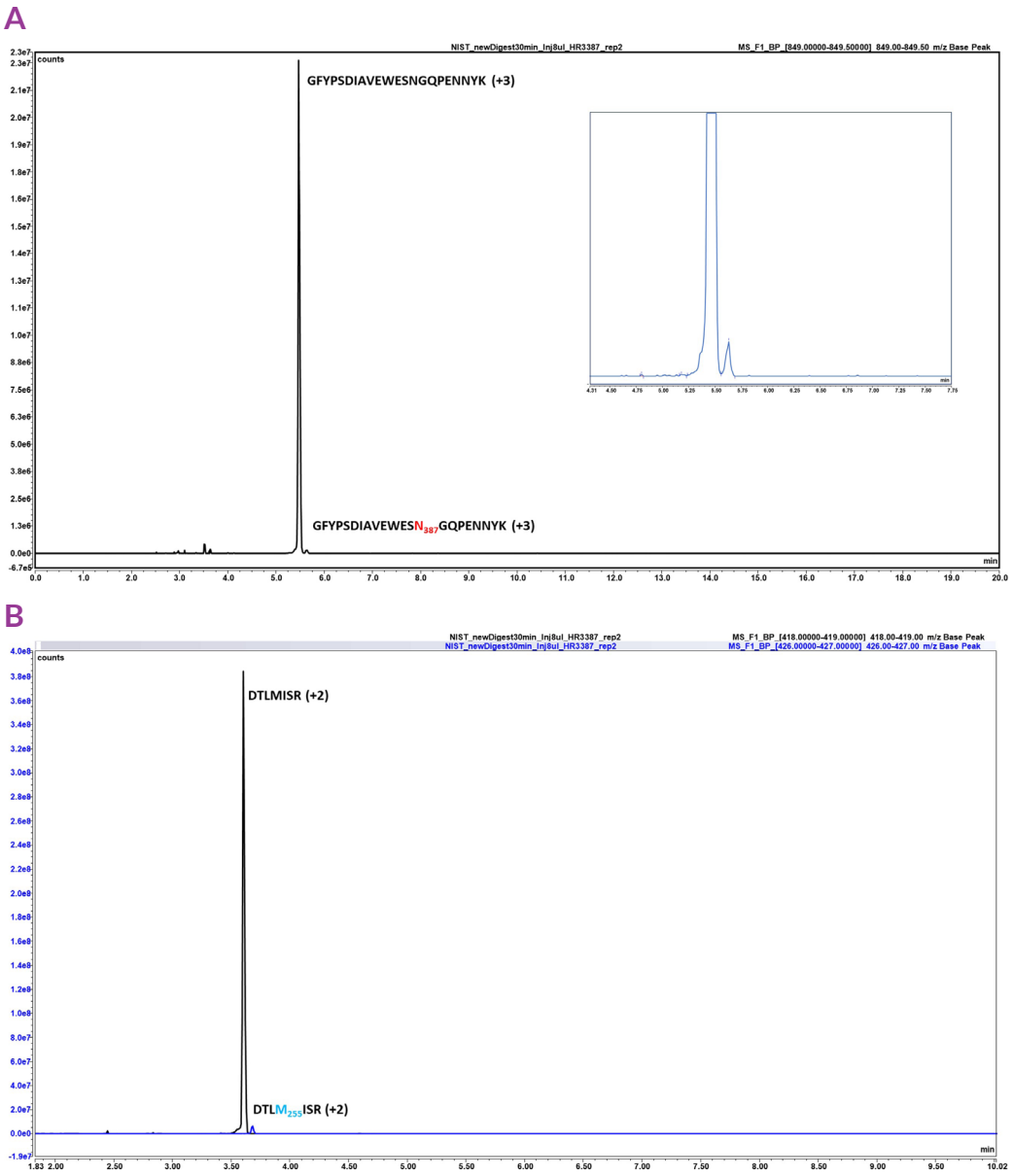



*Figure 3 – A) Extracted ion electropherogram (XIE) for PENNYK peptide unmodified and deamidated form (charge state +3). Mass range used was between 849.0 and 849.5 m/z. B) Overlay of the XIE for DTLMISR peptide (black) and its oxidized form (blue).*

## References

1. Dykstra A.B., Flick T.G., Lee B., *et al.* Chip-Based Capillary Zone Electrophoresis Mass Spectrometry for Rapid Resolution and Quantitation of Critical Quality Attributes in Protein Biotherapeutics *J. Am. Soc. Mass Spectrom.* (2021), 32, 8, 1952–1963.
2. Millán-Martín, S., Jakes, C., Carillo, S. *et al.* Comprehensive multi-attribute method workflow for biotherapeutic characterization and current good manufacturing practices testing. *Nat Protoc* (2022). https://doi.org/10.1038/s41596-022-00785-5