Optimized digestion procedure and characterization for monoclonal antibodies and proteins by CESI-MS

Featuring CESI 8000 Plus and TripleTOF® 6600+ LC-MS/MS System

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Peptide mapping by liquid chromatography-mass spectrometry (LC-MS) is a widely utilized technique to characterize monoclonal antibodies (mAb) and protein therapeutics. It employs a bottom-up approach where the mAb of interest is digested with an enzyme, such as trypsin and the resulting peptides are analyzed by tandem mass spectrometry (MS/MS).

Reverse-phase C18 columns are a primary choice for peptide mass mapping of mAb, but characterization of post-translation modifications (PTMs), such as various type of glycosylations and identification of very large hydrophobic[1], and small hydrophilic peptides can be a challenge.[2] Capillary electrophoresis-mass spectrometry (CESI-MS) separates analytes by by their ionic mobility related to the charge/mass ratio that makes it an orthogonal technique to peptide mass mapping by LC-MS.

CESI-MS offers peptide identification with high-sequence coverage (Figure 1) and characterization of multiple PTMs in a single analysis[2,3,4]. CESI-MS operates at nano flow range (~ 40 nL/min), increasing ionization efficiency and reducing ion suppression.

Using a preconcentration technique such as transient isotachophoresis and capillary zone electrophoresis (t-ITP-CZE) enables greater sensitivity due to high sample loading.[4] In addition it increases the assay sensitivity and separation resolution of glycopeptides.[2,4]

- Adalimumab Light chain:

DIQMTQSPSSLSASVGDRVTITCRASQGIRNYLAWYQQKPGKAPKLLIYAASTLQ
SGVPSRFSGSGSGTDFTLTISSLQPEDVATYYCQRYNRAPYTFGQGTKVEIKRTV
AAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVT
EQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

- Adalimumab Heavy chain:

EVQLVESGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQAPGKGLEWVSAITW
NSGHIDYADSVEGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAKVSYLSTASSL
DYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW
NSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK
KVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH
EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC
KVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDI
AVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE
ALHNHYTQKSLSLSPGK

Figure 1. High sequence coverage of light and heavy chains of Adalimumab using the sample prep and optimized conditions provided in this technical note. Green color: high confidence; Orange color: middle confidence

CESI 8000 Plus and TripleTOF® 6600+ System

Further t-ITP-CZE increases the assay sensitivity and separation resolution of glycopeptides.[2,5,6]

In this technical note, we provide a monoclonal antibody and protein digestion procedure specific for CESI-MS application, since it is very important to obtain a high sequence coverage of mAb and biotherapeutic proteins.

Furthermore, we summarized a good practice for peptide analysis by CESI-MS.

Key Features of CESI 8000 Plus coupled to TripleTOF® 6600+ System

- High sequence coverage of monoclonal antibody or protein based biotherapeutics
- Electrophoretic separation of various glycopeptides
- Minimum sample used (nanoliters) to obtain high sensitivity providing identification of low level PTMs.
- MS/MS confirmation of short, very large hydrophobic, and small hydrophilic peptides often missed in LC-MS

Methods

Equipment

Temperature adjustable water bath or thermoshaker (37 °C to 50 °C), speedVac. dryer, CESI 8000 system, OptiMS BFS Capillary (SCIEX, B07362), CESI adapter (P/N B07366), SCIEX mass spectrometer with NanoSpray® Ion Source III (TripleTOF® 6600+ System), CESI vials (SCIEX, B11648), CESI caps (SCIEX, B24699), PCR microvials (SCIEX, 144709), Nanovials (SCIEX, 5043467).

Reagents

RapiGest SF (Waters, 186008090), Trypsin Gold (Promega, V5280), Ammonium bicarbonate (AmBic, Millipore Sigma, A6141), Dithiothreitol (DTT, Promega, V3155), Iodoacetamide (IAA, Millipore Sigma, I1149), Acetic acid, glacial (HAc, Millipore Sigma, A6283), Formic acid (MS-grade, Fisher-Scientific, A117-50), 7.5 M Ammonia Acetate (Millipore Sigma, A2706), 5 M Sodium Chloride (NaCl, Millipore Sigma, S6546), 1 mol/L (1 N) Hydrochloric acid (HCl, Millipore Sigma, 1090571000), Methanol (MS-grade, Millipore Sigma, 900688), tuning solution part of the TripleTOF 6600+ system installation kit (SCIEX, 4456736).

Reagent preparation

500 mM dithiothreitol (DTT): weight 77 mg in a microcentrifuge tube and add 1 mL of DDI water.

500 mM iodoacetamide (IAA): weight 92 mg IAA in a microcentrifuge tube and add 1 mL of DDI water. Solution should be made fresh each time and kept in dark.

500 mM ammonium bicarbonate (AmBic): weight 3.95 g into a volumetric flask and adjust with DDI water to 100 mL.

Enzyme preparation (Gold Trypsin): reconstitute Trypsin with 50 mM acetic acid to a concentration of 1 µg/µL.

20% acetic acid (HAc): add 10 ml of HAc into 40 mL of DDI water.

0.1 M NaOH add 1 mL of NaOH to 49 mL of DDI water.

0.1 M HCl add 5 mL of HCl to 45 mL of DDI water.

Leading and background electrolytes (LE and BGE)

LE (200 mM ammonium acetate pH 4): Make 50 mL 400 mM ammonium acetate (AmAc) solution (Add 2.7 mL of 7.5 M AmAc into 47.3 mL of DDI water) and adjust the pH of the solution to 4.0 by adding aliquots of 20% HAc freshly prepared. Adjust the volume of final solution to 100 mL and mix the solution by inverting the flask 3 times. It can be store at 2 °C to 8 °C up to 24 months.

BGE (10% (v/v) HAc): Add 2.0 mL of HAc into the 18 mL of DDI water and mix it well. Sonicate solution at least 15 min prior to use. **IMPORTANT:** This solution must be prepared fresh every day.

Tips for optimal results:

1. **Avoid using non-volatile salts** because it might result in plugged sprayer tips.
2. **Avoid using extremely low conductivity buffers** because they mighty result in sprayer tip breakages and side leaks.
3. **Avoid using BGE's with high ionic strength** (e.g. 10% Formic acid) to keep the separation current lower than 5 µA as it might cause permanent damage to the separation capillary and its coating.

Sample preparation prior digestion

mAb digestion buffer: Add 200 µL of 500 mM ABC into RapiGest SF vial for a final concentration of 0.5 µg/mL

mAb sample: Add 100 µg of selected mAb into a 1.5 mL microcentrifuge tube, add 20 µL of mAb digestion buffer, then add 60 µL DDI water to make a final volume of 80 µL.

TIP: The surfactant RapiGest SF breaks-down into non-interfering and non-ion suppressing products by acidifying final sample mix.

TIP: Therapeutic proteins or mAbs with higher hydrophobic characteristic, might require a higher amount of the RapiGest SF.[3]

Sample digestion

mAb reduction and alkylation

Step 1. Add 4 µL of 0.5 M DTT to mAb sample for a final concentration of 10 mM DTT and mix the sample mixture well by quick vortexing

Step 2. Incubate the sample at approx.+56 °C for 45 minutes

Step 3. Cool to room temperature for 10 minutes

Step 4. Add 4 µL of 0.5 M IAA into the sample mixture and incubate the sample in dark for 30 minutes

Step 5. To quench the alkylation reaction, add 2 µL of 0.5 M DTT into the sample mixture

Monoclonal antibody and protein digestion

Step 1. Prior to enzyme digestion, add 5 µL of DDI water for a final volume of 95 µL in sample mixture

Step 2. Add 5 µL of reconstituted trypsin for a final 1:20 enzyme-to-protein sample ratio

Step 3. Finger flick the sample tube to mix

Step 4. Incubate the sample mixture at the approx.. +37ºC for 2 hours

Step 5. After digestion, quick spin the sample tube for 10 seconds

Step 6. Add 1 M formic acid to the sample mixture for a final concentration of 0.5% (11 µL of 1 M formic acid to 100 µL sample reaction mixture)

IMPORTANT: Other proteases can be used for mAb or protein digestion; the protein/enzyme ratio and/or digestion time will need to be adjusted adjust depending on enzyme manufacturer instructions.

Tips for digested sample storage:

- **For digest in solution:** Aliquot 20 µL of sample mixture, add 40 µL of LE buffer pH 4.0. This sample may be stored at 2-8 ºC for one week. Centrifuge and remove pellet prior to analysis as described in critical step below.
- **For lyophilized digest:** Dry 100 µg of digest using a centrifugal vacuum concentrator. Store the digest at -15 to -30 ºC for approx. half a year..

Table 1. CESI cartridge conditioning

	Event	Time (min)	Pressure (psi)	Direction	Solution/Note
1	Rinse	10	100	Forward	Methanol/emitter dip in methanol
2	Rinse	3	100	Reverse	Methanol emitter dip in methanol
3	Rinse	10	100	Forward	DDI water/emitter dip in DDI water
4	Rinse	3	100	Reverse	DDI water/emitter dip in DDI water
5	Rinse	10	100	Forward	0.1 M NaOH/emitter dip in DDI water
6	Rinse	10	100	Forward	0.1 M HCl/emitter dip in DDI water
7	Rinse	10	100	Forward	BGE/emitter dip in DDI water
8	Rinse	3	100	Reverse	BGE emitter dip in DDI water

Sample preparation prior CESI-MS

Monoclonal antibody used in this case was adalimumab. Digested mAb sample was diluted in a 1:2 (*v:v*) ratio with 200 mM ammonium acetate at pH 4.

It is generally recommended to have final LE concentration of 100 mM ammonium acetate pH4 in the sample. In a few cases, The higher concentration of LE might help focusing the sample especially when lower separation voltage is used.

To adjust sprayer position a pI marker (pI 9.5, 5 µL plus 95 µL of BGE) was infused. Further instructions can be found in BFS CESI manual, chapter 5.[6]

IMPORTANT: Every sample that is mixed with LE needs to be centrifuged for at least 5 min at 14 000 rpm, to avoid cartridge clogging by precipitant.

CESI

For the peptide mapping separation, we used a Bare-Fused Silica Surface OptiMS (BFS) Cartridge.

OptiMS cartridge conditioning:

Prior to use, the BFS cartridge was conditioned following a series of rinses as described in Table 1.

Adjusting the sprayer position:

To adjust sprayer position a pI marker (pI 9.5, 5 µL plus 95 µL of BGE) was used. Sprayer position should be at least 2-3 mm from the orifice to avoid tip clogging due to temperature and voltage applied.Further instructions can be found in BFS CESI manual, chapter 5.[4]

Table 2. CESI separation conditions used for the peptide mapping

	Event	Time (min)	Pressure (psi)	Voltage (kV)	Direction	Solution/Note
1	Rinse	2.5	100		Forward	0.1 M NaOH
2	Rinse	2.5	100		Forward	0.1 M HCl
3	Rinse	4	100		Forward	DDI water
4	Rinse	3	75		Reverse	10% Acetic acid
5	Rinse	4	100		Forward	10% Acetic acid
6	Injection	60 s	5		Forward	10% Acetic acid
7	Wait	0	0		Forward	DDI water
8	Injection	25 s	0.5		Forward	10% Acetic acid
9	Separation	40	0	20	Forward	10% Acetic acid
10	Separation	5	0	1	Forward	5min ramp down

The starting ion spray voltage and sprayer position needs to be optimized per each cartridge and can vary from 900 to 1 800 V. In addition, Sprayer position should be at least 2 mm from the orifice to avoid tip clogging due to temperature and voltage applied.

Table 3. CESI overnight and over weekend storage

	Event	Time (min)	Pressure (psi)	Direction	Solution/Note
1	Rinse	5	100	Forward	0.1 M NaOH
2	Rinse	5	100	Forward	0.1 M HCl
3	Rinse	5	100	Reverse	DDI water
4	Rinse	999	5	Forward	DDI water

Table 4. CESI long time storage

	Event	Time (min)	Pressure (psi)	Direction	Solution/Note
1	Rinse	5	100	Forward	0.1 M NaOH
2	Rinse	5	100	Forward	0.1 M HCl
3	Rinse	5	100	Reverse	DDI water
4	Rinse	5	100	Forward	DDI water
5	Vacuum	10	100	Forward	Dry (Storage)
6	Vacuum	10	100	Reverse	Dry (Storage)

CESI-MS separation conditions:

The Adalimumab digest sample in LE was injected hydrodynamically for 60 s at five psi, resulting in an injection volume of approx. 44 nL, corresponding to a sample load of 39 ng. Table 2 describes the separation conditions for the peptide mapping method.

TIP: It is recommended to lower the separation voltage to allow for better separation of glycopeptide species that are difficult to resolve (Figure 2). Example, for separation voltage of 15 kV, the separation analysis time is 50 minutes.[2]

IMPORTANT: Generally, it is not recommended to apply a separation voltage across the BFS cartridge that exceeds 20 kV.

After use and to increase capillary life, it is recommended to use the method described in table 3. This method allows over 16 hours of a gentle and continuous flow of DDI water through the separation capillary keeping it from clogging while on board the instrument. If the capillary cartridge won't be used for longer than overnight period, it is recommended to be stored dry by following the steps in table 4.

Mass spectrometry

The TripleTOF® 6600+ System was coupled with the NanoSpray® Ion Source III with CESI adapter and controlled by Analyst® Software 1.8.1 . The peptide mapping of adalimumab was performed using data dependent acquisition (DDA) with 10 MS/MS cycles (150 and 50 ms accumulation times for MS and MS/MS, respectively). The acquisition mass range was from 350 to 2250 m/z with cycle time of 0.8 s. The precursors with charges from +2 to +5 and intensity above 100 cps were triggered for MS/MS fragmentation selected precursors were fragmented using a rolling collision energy (CE) with spread of ± 5 V. Source parameters are listed in the Table 5. The starting ion spray voltage and sprayer position needs to be optimize per each cartridge.

Separation performance of the cartridge and instrument calibration can be checked using standards. To set up the autocalibration follow the guidelines in CESI manual, chapter 5.[4]

IMPORTANT: A steady flow of ions through the sheathless interface is critical for a stable spray and reproducible separation, which can be accomplished by using volatile reagents such as acetic acid.

Table 5. Summary of mass spectrometry parameters.

Parameter	Value	Parameter	Value
MS mass range	*350-2250 m/z*	*MS/MS mass range*	150-2250 *m/z*
MS accumulation time	*150 ms*	*MS/MS accumulation time*	*50* ms
Curtain gas:	*5 psi*	*Source temperature:*	50 *°C*
Polarity:	+	*Ion spray voltage:*	900-1800 V

Data processing: ProteinPilot™ and BPV Flex Software was used for mAb characterization and SCIEX OS-Q Software were used for peptide mapping analysis.

Results and discussion

CESI-MS peptide mapping

CESI-MS is an effective and complementary technology to LC-MS for the characterization of mAbs at the peptide level with a high sequence coverage. In the example case, we confidently map at least 99% of adalimumab sequence (Figure1). Additionally, trypsin digestion protocol was tested on cetuximab[3], transtuzumab[4,7], bevacizumab and IgGs (data not shown, for detailed study of cetuximab and transtuzumab check reference 3, 4 and 7). Due to high sequence coverage, micro-

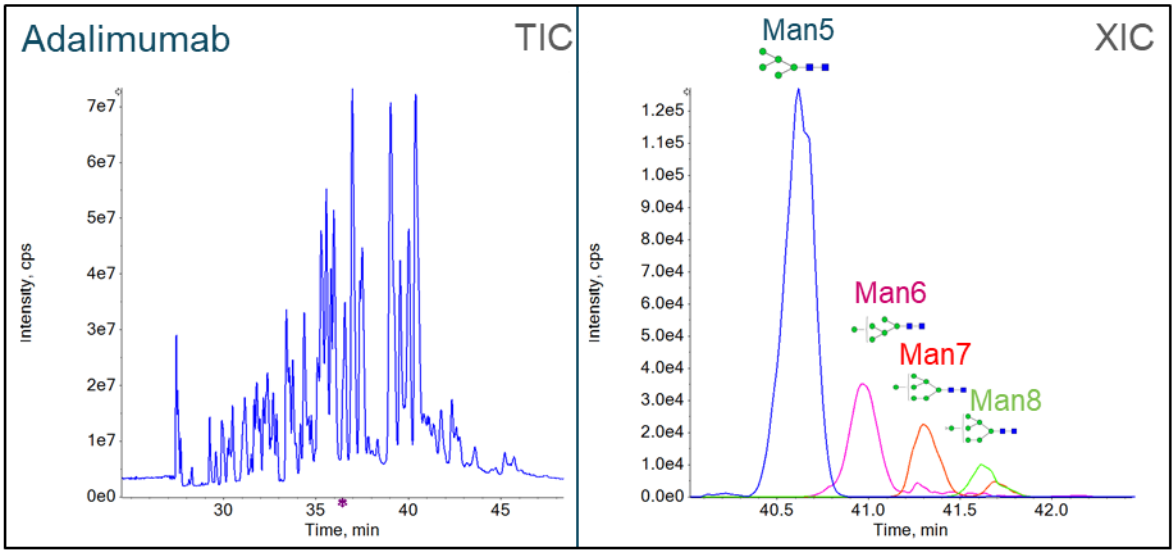

Figure 2. Characterization of adalimumab by CESI-MS. Left panel: Total ion chromatogram (TIC) of adalimumab digest; Right panel: Extracted ion chromatograms (XICs) of low abundant high mannose N-glycans on aspartic acid 301 of EEQYNSTYR peptide. Further information can be found in comprehensive analysis of low abundant mannose glycopeptides in peptide mapping of adalimumab, RUO-MKT-02-11834-A.[2]

sequence changes between different biosimilars could be studied (data not shown, for detailed study check reference 7).[8]

Post translation modifications

While performing peptide mass mapping of adalimumab various glycopeptides were confirmed (data and further information are listed in the technical note. Comprehensive analysis of low abundant mannose glycopeptides in peptide mass mapping of adalimumab)[2], as example high mannose are shown in Figure 2. While glycans are important post-translational modifications that are expected on adalimumab, there are also other PTMs which a can affect the safety, efficacy and even solubility of the biotherapeutic.

Figure 3, illustrates example of the separation between unmodified vs. deamidated and oxidized form of three different peptides of adalimumab. The deamidated peptide FNWYDGVEVHNAK and YNRAPYTFGQGTK were determined to be 3.2% and 2.9% of the total amount of peptide in the sample, respectively. The peptide NSLYLQMNSLR contains 8.6% of oxidized from total amount of peptide. Deamidation and oxidation are the most common PTMs occurring on therapeutic proteins produced using recombinant DNA technology.

Conclusions

Peptide mass mapping remains a key analytical methodology to characterize biotherapeutics from drug discovery through production and it provides the ability to assign specific locations of product attributes. It allows monitoring of amino acid substitution as well as post-translation modifications that are critical for a biotherapeutic's safety and efficacy.

CESI-MS peptide mass mapping is a valuable orthogonal technology that provides additional sequence coverage

compared to LC-MS. In this study, we presented peptide mapping of adalimumab with >99% sequence coverage from ~40 ng sample.

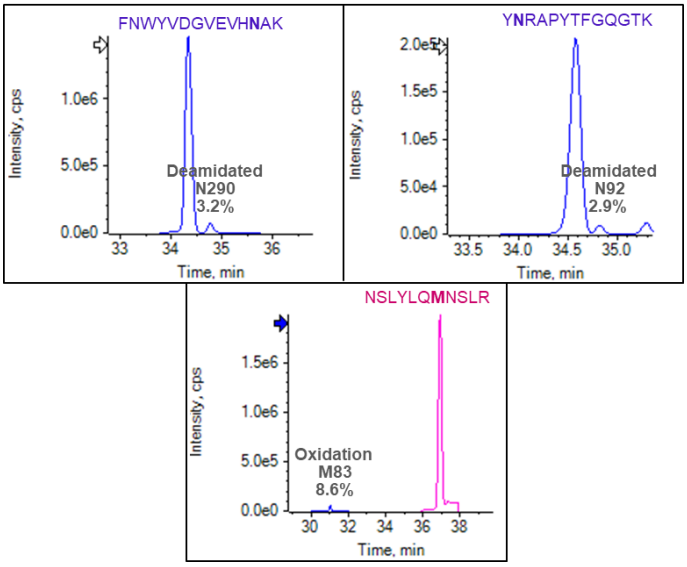

Figure 3. Deamidation of aspartic acids and oxidation of methionine in Adalimumab. Peptide FNWYDGVEVHNAK and YNRAPYTFGQGTK were determined to be 3.2% and 2.9% of the total amount of peptide in the sample, respectively. From total amount of peptide NSLYLQMNSLR 8.6% contains oxidation of methionine.

Furthermore, critical quality attributes, such as glycosylation, deamidation and oxidation of peptides, can be characterize during the peptide mapping analysis without additional measurements.[3,4,8]

References

1. Improving the detection limits for highly basic neuropeptides using CESI-MS, RUO-MKT-02-7150-A, https://sciex.com/Documents/tech%20notes/technology/CESI/Neuropeptide-detection-CESI-MS.pdf

2. Comprehensive analysis of low abundant mannose glycopeptides in peptide mapping of adalimumab, RUO-MKT-02-11834-A

3. Gahoual R, Biacchi M, Chicher J, et al. Monoclonal antibodies biosimilarity assessment using transient isotachophoresis capillary zone electrophoresis-tandem mass spectrometry. MAbs. 2014;6(6):1464-1473.

4. Gahoual R, Burr A, Busnel JM, et al. Rapid and multi-level characterization of trastuzumab using sheathless capillary electrophoresis-tandem mass spectrometry. MAbs. 2013;5(3):479-490.

5. Yu, Ying-Qing, et. al. A complete peptide mapping of membrane proteins: a novel surfactant aiding the enzymatic digestion of bacteriorhodopsin. Rapid Comm in Mass Spectrom. Letter to the Editor. 2004; 18; 711-715.

6. CESI 8000 High Performance Separation System, User manual, RUO-IDV-05-3897-C March 2020 https://sciex.com/Documents/manuals/cesi8000_uManual_B11949AD.pdf

7. Lew, C., Gallegos-Perez, J.L., Fonslow, B., Lies, M. and Guttman, A., 2015. Rapid level-3 characterization of therapeutic antibodies by capillary electrophoresis electrospray ionization mass spectrometry. *Journal of chromatographic science*, *53*(3), pp.443-449.

8. Gahoual R, Busnel JM, Beck A, François YN, Leize-Wagner E. Full antibody primary structure and microvariant characterization in a single injection using transient isotachophoresis and sheathless capillary electrophoresis-tandem mass spectrometry. Anal Chem. 2014;86(18):9074-9081.

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