Intact LC-MS analysis and peptide mapping of recombinant adeno associated virus (rAAV) capsid proteins

Featuring the ZenoTOF 7600 system and the Biologics Explorer software from SCIEX

Haichuan Liu and Zoe Zhang
SCIEX, USA

This technical note describes sensitive and easy-to-adopt LC-MS workflows for intact analysis and peptide mapping of rAAV capsid proteins. The ultra-high MS/MS sensitivity offered by the Zeno trap resulted in nearly complete sequence coverage of capsid proteins in a single injection and confident identification of very low abundant modifications despite limited sample quantity. The unique capability of electron activated dissociation (EAD) allowed differentiation between aspartic (Asp) and isoaspartic acid (isoAsp) isomers of deamidation.

rAAVs are widely used vectors in gene therapy because of being non-pathogenic to humans, possessing low immunogenicity and offering long-term gene expression. rAAVs consist of 3 viral proteins (VPs: VP1, VP2 and VP3) assembled in a 1:1:10 ratio. The characterizations of these VPs and their modifications[1], such as N-terminal acetylation, deamidation and phosphorylation are critical to ensure high product quality of rAAVs. Specifically, deamidation was found to impact transduction efficiency of AAV vectors.[2] However, it is challenging to perform in-depth characterization of VPs in practice due to limited sample quantity, which hampers method optimization and detection of low abundance species. Additionally, it is not possible to differentiate isomers of deamidation using traditional MS/MS techniques such as collision-induced dissociation (CID) and low-energy electron-based dissociation (ExD).

In this technical note, an intact and a peptide mapping workflow with easy-to-use analytical flow methods were employed to characterize a rAAV serotype 8 (rAAV8). The highly sensitive data dependent acquisition (DDA) method with Zeno trap enabled, provided nearly complete sequence coverage of VPs and confident identification of low abundant modifications, such as deamidated species with relative abundance as low as ~0.04%. The differentiation of deamidation isomers was further accomplished by EAD.

Key features of the ZenoTOF 7600 system for rAAV characterization

- **High quality intact data**: Excellent data quality, reproducibility and mass accuracy for intact and subunit analysis, supporting the development of rAAVs
- **Highly sensitive data DDA for peptide mapping**: The Zeno trap provides 5–10-fold increase in detection of fragments, leading to complete sequence coverage and confident identification of extremely low-abundance modifications of rAAVs in limited quantity
- **Powerful EAD for differentiation of isomers**: EAD possesses the unique capability of differentiating isomers of Asp/isoAsp and isoleucine/leucine[3–5]
- **Streamlined and easy-to-use**: Automated data acquisition using SCIEX OS software and data analysis using optimized workflow templates in the Biologics Explorer software enable routine characterization of VPs
- **Powerful tools for result visualization and review**: The Biologics Explorer software offers an array of tools for reviewing and displaying the results

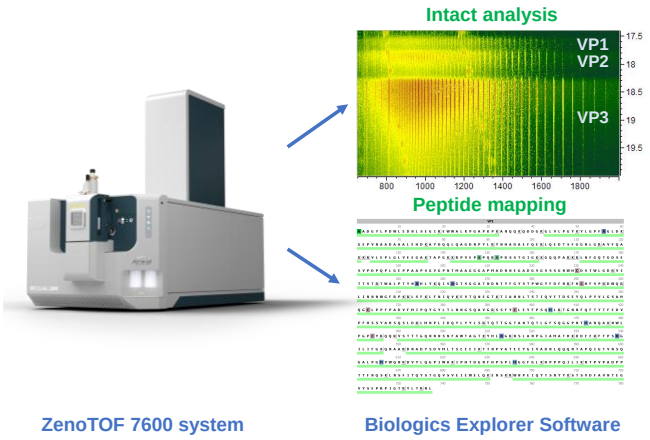

Figure 1. Intact analysis and peptide mapping using the SCIEX ZenoTOF 7600 system and SCIEX Biologics Explorer software. The ZenoTOF 7600 system offers sensitivity, mass accuracy and reproducibility to obtain high quality intact and peptide mapping data. The data analysis is streamlined using default templates in the Biologics Explorer software.

Methods

Sample preparation: The rAAV serotype 8 samples (rAAV8) were purchased from SignaGen Laboratories and Vigene Biosciences. Each vial (30 µL) of SignaGen rAAV8 contains ~1 × 10^{13} genome copies per mL (GC/mL), which is equal to ~3 × 10^{11} viral particles per vial. For intact analysis, the rAAV8 sample was diluted in water and directly analyzed by LC-MS. For peptide mapping, the sample was denatured by guanidine-hydrochloride, reduced with dithiothreitol and alkylated using iodoacetamide, followed by buffer exchange using the Bio-Spin columns (Bio-Rad) and enzymatic digestion (2 hr, 37ºC) using trypsin (Promega). In the heat stress experiment, 1 vial (50 µL) of Vigene rAAV8 sample (1.75 × 10^{13} GC/mL) was incubated at 37ºC for 24 hrs. The stressed sample was digested using the same procedures as above.

Chromatography: The separation of the intact viral proteins (VP1, VP2 and VP3) was performed using an ACQUITY UPLC protein BEH C4 column (2.1 mm × 50 mm, 1.7 µm, 300 Å, Waters) with a 40 minute method (20% B at 0–2 min, 20% to 40% B from 2–32 min, 90% at 33–36 min and 20% at 36.1–40 min). The injection volume for intact analysis was set to 10 µL equaling ~0.3 µg of protein on column.

The tryptic peptides were separated with the gradient displayed in Table 1 using an ACQUITY CSH C18 column (2.1 × 150 mm, 1.7 µm, 130 Å, Waters). A flow rate of 0.25 mL/min was used for all the separations mentioned above. The columns were kept at 60ºC in the column oven of an ExionLC AD system (SCIEX). The mobile phases A and B consist of 0.1% formic acid in water and 0.1% FA in acetonitrile, respectively. For Zeno CID 20 µL of the digest from the SignaGen sample were injected, which equals to 0.2 µg on column, assuming a 60% recovery throughout the sample preparation. In case of Zeno EAD 10 uL of the digest from the Vigene Biosciences sample were injected, equaling 0.6 µg on column when assuming a 60% recovery.

Table 1. LC gradients for peptide mapping of rAAV8.

Time [min]	A [%]	B [%]
Initial	*98*	*2*
2	*98*	*2*
62	*60*	*40*
63	*10*	*90*
66	*10*	*90*
67	*98*	*2*
69	*98*	*2*
70	*10*	*90*
73	*10*	*90*
74	*98*	*2*
80	*98*	*2*

Mass spectrometry: LC-MS data was acquired in the SCIEX OS software using the ZenoTOF 7600 system. The key TOF-MS settings for intact analysis and DDA parameters for peptide mapping are listed in Table 2 and 3, respectively.

Table 2. TOF-MS parameters for intact analysis.

Parameter	Value
Spray voltage	*5500 V*
TOF start mass	*500 m/z*
TOF stop mass	*3000 m/z*
Accumulation time	*0.5 s*
Source temperature	*450ºC*
Declustering potential	*200 V*
Collision energy	*12 V*
Time bins to sum	*40*

Table 3. DDA parameters for peptide mapping using CID.

Parameter	Value
IDA criteria	*Peptide*
Maximum candidate ions	*10*
Charge state	*2 to 10*
Isotope to select	*Most intense*
Exclude time	*6 s after 2 occurrences*
Dynamic CE for MS/MS	*True*
TOF start mass	*100 m/z*
TOF stop mass	*2000 m/z*
Zeno trap	*ON*
Accumulation time	*0.06 s*
Q1 resolution	*Unit*

The deamidated species in the heat-stressed sample were characterized by a targeted approach using EAD MRM^{HR}. The key EAD parameters are provided in Table 4.

Table 4. EAD MRM^{HR} parameters.

Parameter	Value
Electron KE	*7 eV*
Accumulation time	*0.5 s*
Electron beam current	*5000 nA*
Zeno trap	*ON*
ETC	*100%*
Reaction time	*30 ms*

Data processing: Intact and peptide mapping data were processed using the default workflow templates in the Biologics Explorer software. The MRM^{HR} data were analyzed by enabling annotation of *c*+57/*z*–57 fragments in the Biologics Explorer software for differentiation of deamidation isomers.

Intact analysis of rAAV8 capsid proteins

Figure 2 displays the result of the LC-MS measurement of intact rAAV8 capsid proteins (VP1, VP2 and VP3). The ion map of the Biologics Explorer software (Figure 2A) provides excellent visualization of 3 distinct peak series that correspond to VP1–VP3, respectively, including an indication of their signal intensity. The intact protein mapping against the theoretical sequence revealed that the dominant species detected in VP1 and VP3 samples showed a + 42 Da mass shift, which can be attributed to acetylation (Figure 2B and 2D), while VP2 did not carry this modification. Another major modification detected for rAAV8 was phosphorylation, resulting in a +80 Da mass shift. In this study, abundant single phosphorylation was observed for acetylated VP1 (Figure 2B) and VP2 (Figure 2C) without acetylation. These results were consistent with those reported for rAAV8 in literature.[1] Besides major species described above, a few low abundant species were detected, including VP3 without acetylation (~0.4%, Figure 2D), singly phosphorylated VP3 with acetylation (~1.5%) and doubly phosphorylated VP2 (~2.3%, Figure 2C), indicating the high sensitivity of this intact workflow. It is worth noting that all the VP peaks detected were measured with very high mass accuracies (<20 ppm), enabling confident assignment of each species.

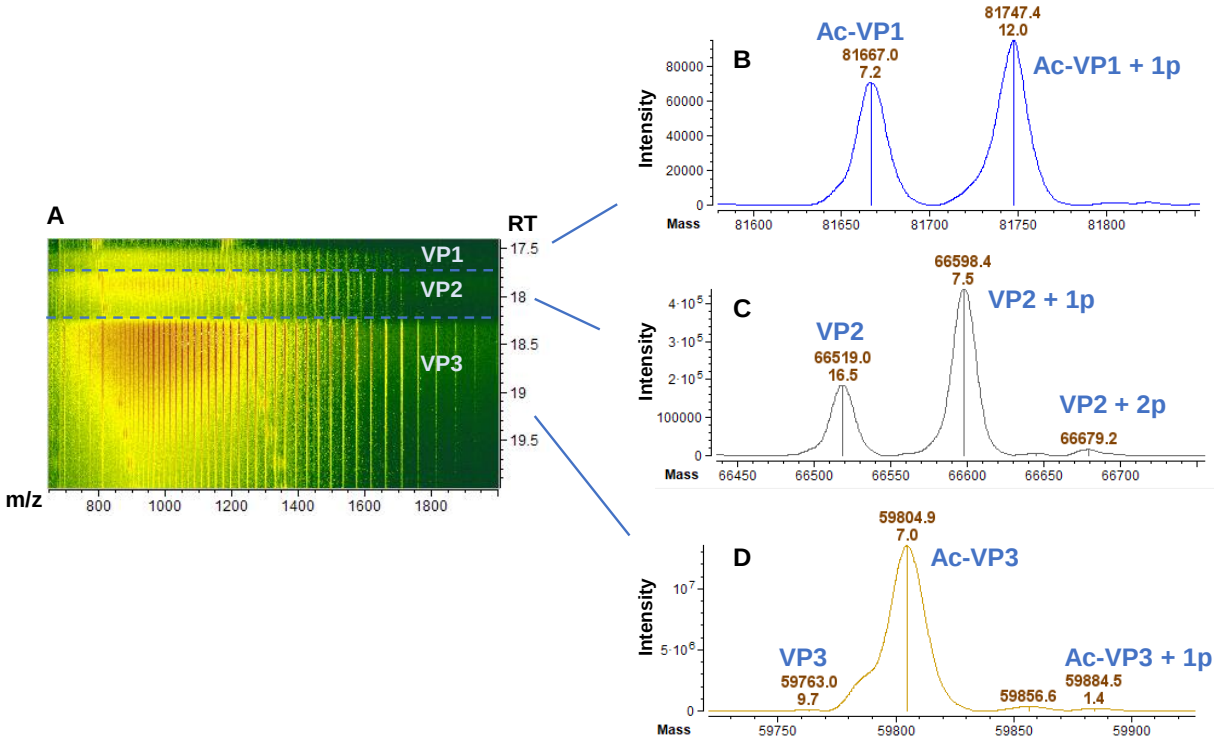

Figure 2. Intact analysis of rAAV8 capsid proteins (VP1–VP3) using the Biologics Explorer software. The ion map (A) of the Biologics Explorer software offers excellent visualization of the VPs separated chromatographically. The protein mapping results confirmed acetylation of VP1 (B) and VP3 (D) and phosphorylation of VP1 (B) and VP2 (C). Minor species, including non-acetylated and singly phosphorylated VP3 (D) as well as doubly phosphorylated VP2 (C), were also detected. Ac = acetyl, p = phosphorylation.

Peptide mapping of rAAV8 VPs

The sequence coverage of rAAV8 capsid proteins was assessed using the theoretical sequence of VP1, which completely covers the amino acid sequences of VP2 and VP3. As displayed in Figure 3, a near complete sequence coverage of VP1 (94.7%) was obtained from single injection of rAAV8 digest using the highly sensitive DDA with CID. Most of the sequences not covered correspond to short tryptic peptides containing ≤5 amino acid residues. These short peptides were not well retained with the reversed phase C18 column. In addition, they predominantly formed singly charged species that were not targeted in this study.

Modifications of rAAV8 VPs

The acetylation at the N-termini of VP1 and VP3 was confidently identified from high-quality CID MS/MS spectra of the 2 corresponding peptides, for example, VP1[1–19] and VP3[1–35] (Figure 4A and 4B). The non-acetylated form of VP3[1–35] in lower abundance was also identified with high confidence (Figure 4C).

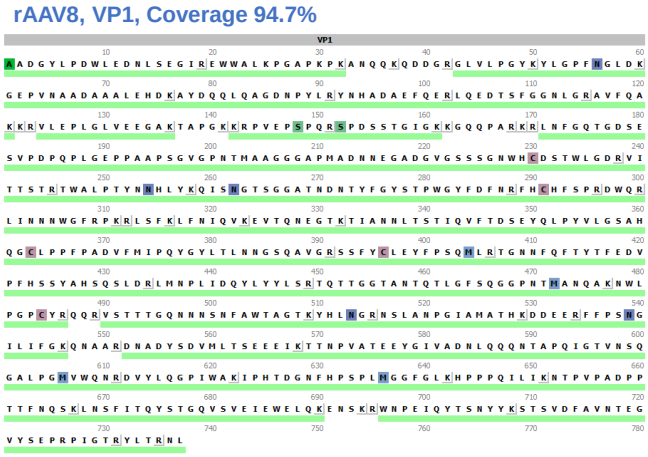

Figure 3. Sequence coverage of rAAV8 VP1. A nearly complete sequence coverage (94.7%) was obtained from a single injection of the trypsin digest of rAAV8 using a highly sensitive DDA CID method.

As reported previously[2], deamidation is critical to the function of AAV. Hence, confident identification of low abundant deamidation is essential to ensuring the quality of AAV vectors. A few deamidated peptides of rAAV8 were identified in this study, such as YLGPFNGLDK, TWALPTYNNHLYK and FFPSNGILIFGK. The first peptide corresponds to VP1[51–60] while other 2 peptides are found in all 3 VPs. Figure 5 displays the extracted ion chromatograms (XICs) of native and deamidated species of YLGPFNGLDK and FFPSNGILIFGK. The deamidated forms of these 2 peptides were present at very low abundance, as low as ~0.9% for YLGPFNGLDK (Figure 5A) and ~0.04% for FFPSNGILIFGK (Figure 5B). This combined with limited sample quantity makes it very challenging to detect and confidently identify these deamidated species. However, the high MS/MS sensitivity offered by the Zeno trap allowed confident identification of these species (Figure 6). In addition, the location of deamidation in these peptides can be accurately determined based on the *m/z* of y_4/y_5 for YLGPFNGLDK (Figure 6A–6C) and y_7/y_8 for FFPSNGILIFGK (Figure 6D–6F).

Another challenge of comprehensive characterization of deamidated peptides is the differentiation between Asp and isoAsp isomers, which may affect protein function.[9] This can be addressed by leveraging the unique capability of EAD for isomer differentiation.[3–5] To enhance deamidation, the rAAV8 sample was thermally stressed at 37ºC for 24 hours. Consequently, 3 major deamidated species of YLGPFNGLDK were observed in the chromatogram, particularly a high-abundance peak at RT= 27.4 minutes (Figure 7A). This result indicates the high susceptibility of rAAV8 upon thermal stress, demonstrating the need to fully characterize deamidation.

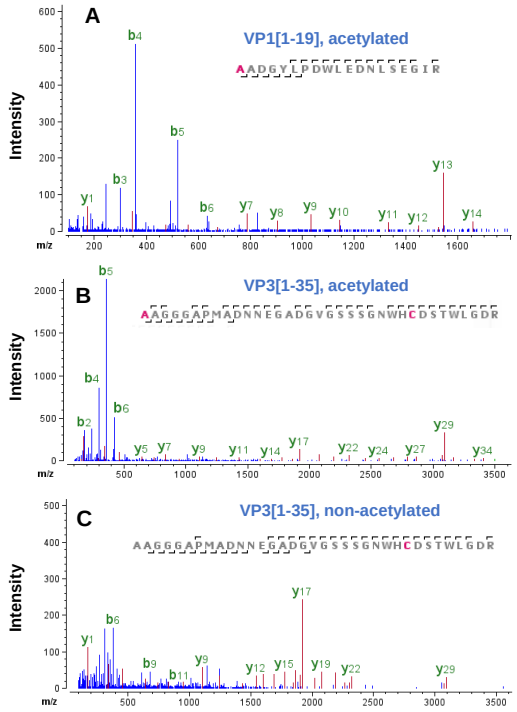

Figure 4. Identification of N-terminal peptides of VP1 and VP3. The high-quality CID MS/MS spectra led to confident identification of acetylated N-terminal peptides of VP1 (A) and VP3 (B). In addition, low abundant VP3[1–35] without acetylation was identified with high confidence.

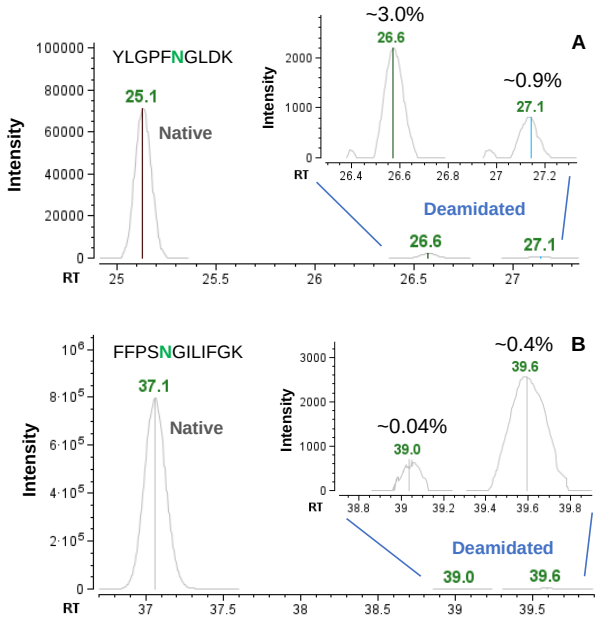

Figure 5. XICs of native and deamidated species identified. A: YLGPFNGLDK and B: FFPSNGILIFGK. The deamidation with abundance as low as ~0.04% was confidently identified using the highly sensitive CID method.

The EAD MS/MS spectra of the deamidated species observed for YLGPFNGLDK revealed that the peaks at RT = 27.4 min and 30.1 min correspond to the isoAsp form based on the detection of its z_5–57 signature fragment (Figure 7 B and 7D). The presence of 2 isoAsp species can be explained by racemization of the naturally occurring *L*- to the much lower abundant *D*-form, as reported previously.[7,8] The peak at RT = 28.0 min was assigned to the Asp isomer due to the detection of a z_5–44 fragment and the absence of a z_5–57 fragment. It was reported previously that the *z* - 44 fragment was a signature fragment of the Asp isomer.[6]

Illustrated in Figure 8 is an even more complex case showcasing the ability of EAD for differentiation between Asp/isoAsp isomers. Deamidation on TWALPTYNNHLYK containing 2 Asn residues in direct proximity to each other may theoretically produce 2 pairs of Asp/isoAsp isomers, 3 of which were detected in this study. According to the *m/z* values of z_5 and z_6 fragments, the peaks at 26.9 min and 28.0 min correspond to deamidation at N8 of TWALPTYNNHLYK, while the peak at 27.4 min is related to deamidation at N9. The former 2 deamidated species were further assigned as the Asp and isoAsp isomers, respectively, based on the detection of their signature fragments (z_6–44 in Figure 8B and z_6–57 in Figure 8D).

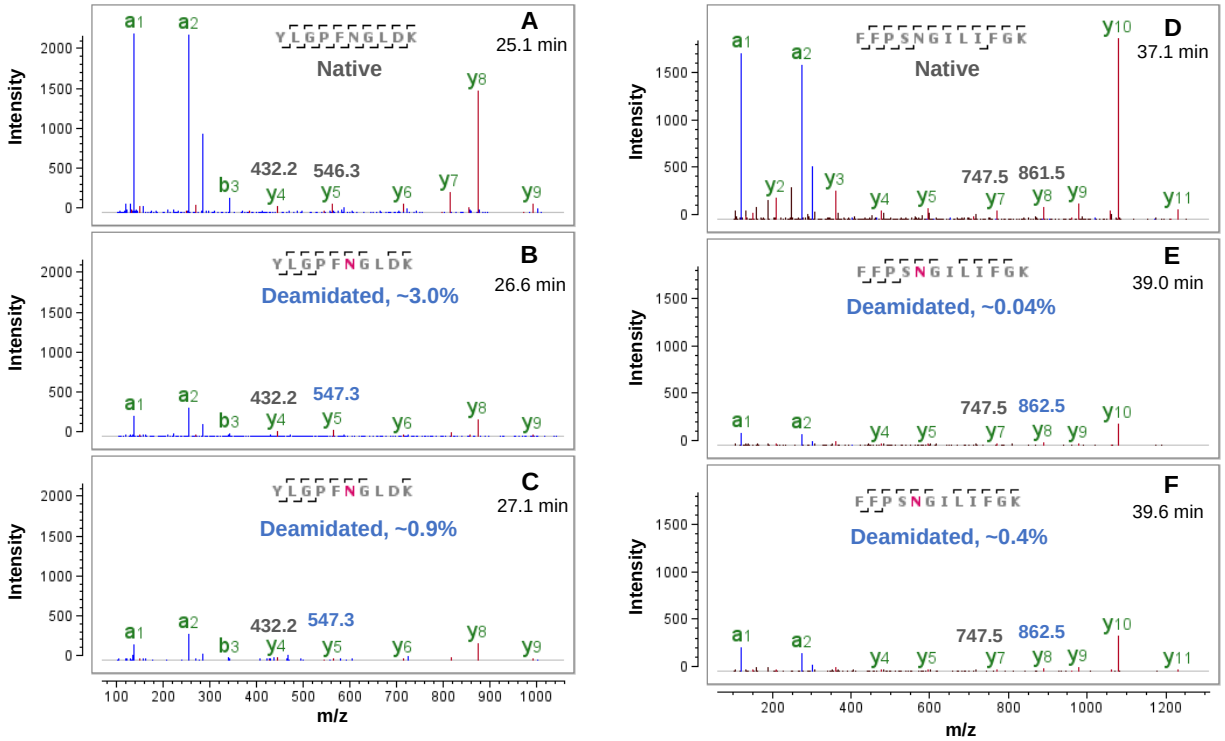

Figure 6. CID MS/MS spectra of native and deamidated species. A–C: peptide YLGPFNGLDK and D–F: peptide FFPSNGILIFGK. Confident identification was achieved for the deamidated forms present at very low level (~0.9% for YLGPFNGLDK and ~0.04% for FFPSNGILIFGK). The *m/z* values of the signature fragments (y_4/y_5 for YLGPFNGLDK and y_7/y_8 for FFPSNGILIFGK) that indicate the position of deamidation were shown in each spectrum.

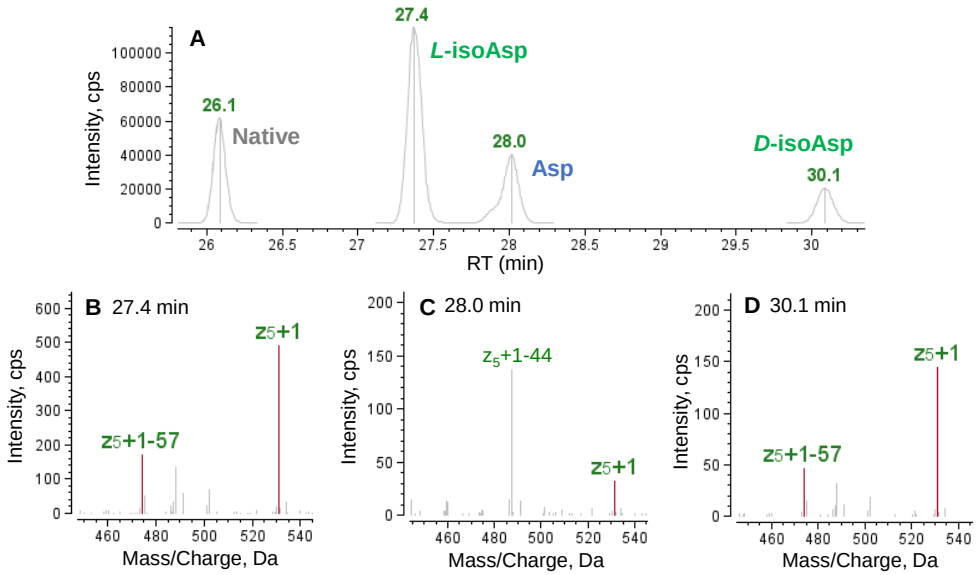

Figure 7. Signature EAD fragments of 3 deamidated species of YLGPFNGLDK (2+). The 3 deamidated species of YLGPFNGLDK in the XIC (A) were identified as the Asp or isoAsp isomer based on the detection of their respective signature fragments, for example z_5–57 for isoAsp (B and D) and z_5–44 for Asp[6] (C). The formation of 2 isoAsp species can be attributed to racemization (from *L*- to *D*-form), as reported previously.[7,8]

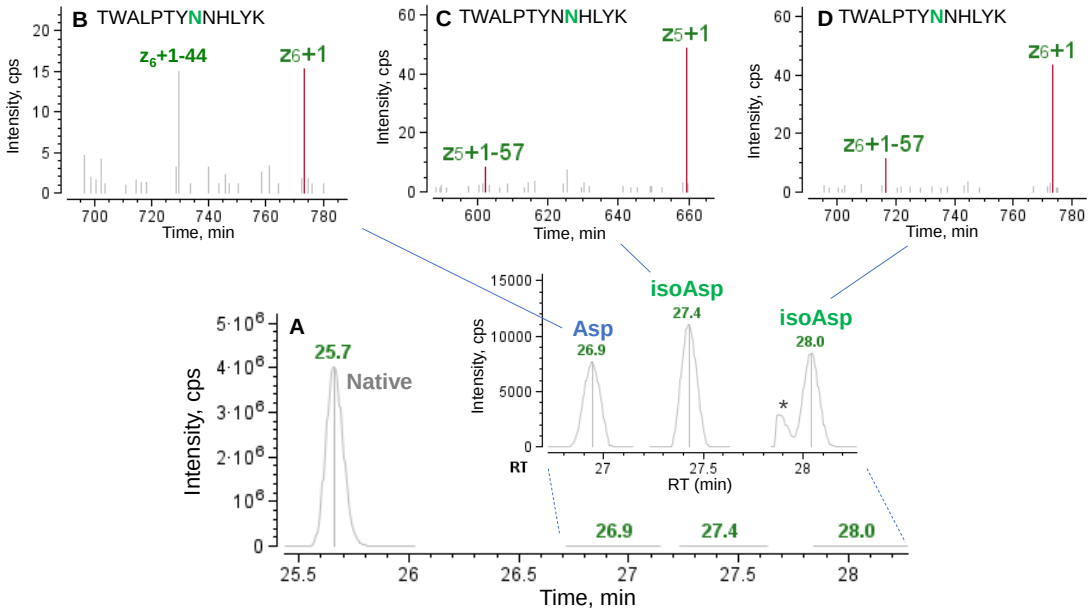

Figure 8. Signature EAD fragments of three deamidated species of TWALPTYNNHLYK (3+). The 2 deamidated species at RT = 26.9 min and 28.0 min (A) can be assigned as the Asp and isoAsp isomers of N8-deamidated TWALPTYNNHLYK, respectively, based on the detection of the signature fragments z_6–44 (B) and z_6–57 (D). The peak at RT= 27.4 minutes corresponds to the isoAsp isomer of N9-deamidated TWALPTYNNHLYK due to the presence of a z_5–57 fragments. *Overlapping isotope of a z = 4+ species not being related to TWALPTYNNHLYK.

Similarly, the deamidated species at 27.4 minutes can be confidently assigned as the isoAsp isomer due to the presence of z_5–57. It is worth noting that the order of elution for Asp/isoAsp isomers is opposite for YLGPFNGLDK (Figure 7A) and TWALPTYNNHLYK (Figure 8A), indicating that one cannot rely solely on retention time to differentiate these isomers. Taken together, the results described above demonstrate the power of EAD for identification and differentiation of deamidation-derived isomers. As deamidation affects the function of rAAVs[2], the ability to differentiate its isomers becomes especially important for proper quality assessment and stability studies of rAAV products.

Phosphorylation is an important PTM that has significant impact on transduction efficiency intracellular trafficking of AAVs.[10] The CID MS/MS spectra of 2 serine phosphopeptides identified in this work were displayed in Figure 9. The detection of phosphate-containing fragments, including b_7 and y_5 for KRPVEPSPQR (Figure 9A) and b_2 for SPDSSTGIGK (Figure 9B), enabled confident identification and localization of phosphorylation.

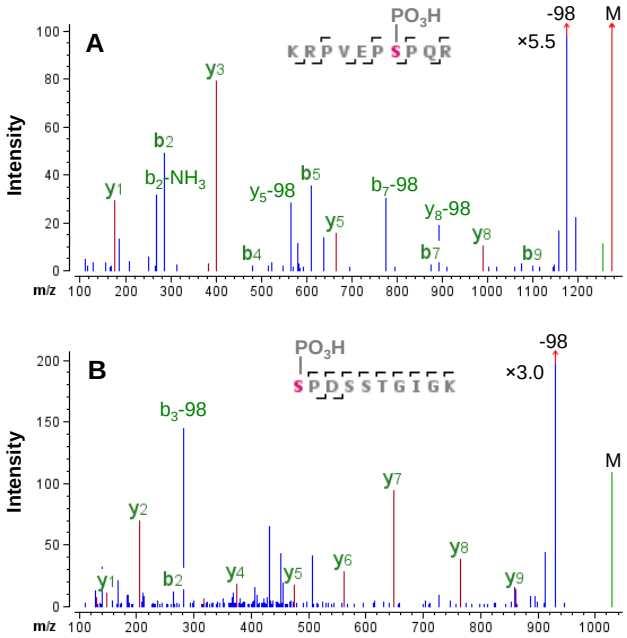

Figure 9. CID MS/MS spectra of 2 serine phosphopeptides. A: KRPVEPSPQR and B: SPDSSTGIGK. The detection of CID fragments b_7 and y_5 for KRPVEPSPQR (A) and b_2 for SPDSSTGIGK (B) allowed confident localization of the phosphate group in these 2 peptides.

Conclusions

- The presence of 3 capsid proteins, acetylation for VP1 and VP3 and phosphorylation for all VPs were confirmed for rAAV8 by high-quality accurate intact mass analysis
- Nearly complete sequence coverage was achieved for rAAV8 VPs in a single injection using the highly sensitive CID method Confident identification of deamidated species at extremely low abundance (as low as ~0.04%) was enabled by excellent MS/MS sensitivity offered by the Zeno trap
- Unambiguous differentiation between Asp and isoAsp isomers, which is critical to quality assessment and stability studies of rAAV products, was enabled by EAD
- Streamlined data analysis was facilitated through templates for intact and peptide mapping with optimized processing settings, as well as powerful visualization tools in the Biologics Explorer software

References

1. Bertin Mary *et al.* (2019) Post-translational modifications in capsid proteins of recombinant adeno-associated virus (AAV) 1-rh10 serotypes. *The FEBS J.* **286**: 4964-4981.
2. April Giles *et al.* (2018) Deamidation of amino acids on the surface of adeno-associated virus capsids leads to charge heterogeneity and altered vector function. *Mol. Ther.* **26** (12): 2848-2862.
3. Differentiation of aspartic and isoaspartic acid using electron activated dissociation (EAD). SCIEX technical note, RUO-MKT-02-12550-B.
4. Differentiation of leucine and isoleucine using electron activated dissociation (EAD). SCIEX technical, note RUO-MKT-02-12605-B.
5. An evaluation of single injection platform method for advanced characterization of protein therapeutics using electron activation dissociation (EAD). SCIEX technical note, RUO-MKT-02-13965-A.
6. Nadezda Sargaeva *et al.* (2009) Identification of aspartic and isoaspartic acid residues in amyloid β peptides, including Aβ 1-42, using electron-ion-reactions. *Anal. Chem.* **81**(23): 9778-9786.
7. Marine Morvan and Ivan Miksik. (2021) Recent advances in chiral analysis of proteins and peptides. *Separations* **8**(8): 112.
8. Superior characterization and monitoring of product quality attributes using an electron activated dissociation (EAD) based multi-attribute method (MAM). SCIEX technical note, RUO-MKT-02-14045-A.
9. Hiroaki Sakaue *et al.* (2017) Isomeric replacement of a single aspartic acid induces a marked change in protein function: the example of ribonuclease A. *ACS Omega* 2(1): 260-267.
10. Li Zhong *et al.* (2008) Tyrosine phosphorylation of AAV2 vectors and its consequences on viral intracellular trafficking and transgene expression. *Virology* 381(2):194-202.

SCIEX

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices