Superior characterization and monitoring of product quality attributes using an electron activated dissociation (EAD)-based multi-attribute method (MAM)

Featuring the ZenoTOF 7600 system and Biologics Explorer software from SCIEX

Haichuan Liu[1], Ricardo Gomes[2, 3], Zoe Zhang[1]
[1]SCIEX, USA; [2]iBET, Instituto de Biologia Experimental e Tecnológica, Portugal; [3]ITQB, Instituto de Tecnológia Química e Biológica António Xavier, Universidade Nova de Lisboa, Portugal

This technical note highlights the power of an EAD data-dependent acquisition (DDA) platform method[1-4] for the identification of product quality attributes (PQAs) of a monoclonal antibody (mAb) and their quantification and monitoring using a single injection as part of a multi-attribute method (MAM). The streamlined MAM workflow that links the Biologics Explorer software to compliance-ready SCIEX OS software will also be demonstrated.

MAM is a powerful LC-MS method for simultaneously monitoring and quantifying multiple PQAs or critical quality attributes (CQAs) of a biotherapeutic. Traditionally, CID-based MS/MS is employed as the first step for identification, followed by quantification with either only MS or MS and MS/MS workflows. However, full sequence confirmation, including the differentiation of leucine vs. isoleucine and aspartic vs. isoaspartic acid (Asp vs. isoAsp) for deamidated peptides and the localization of fragile modifications, such as glycosylation, are not possible with CID-based MS/MS methodologies. Hence, the CID-based identification workflow must rely on complementary MS/MS techniques that use more advanced instrumentation in separate experiments before quantification of selected species can be performed. This challenge can be addressed using the EAD platform method[5,6], which enables confident and accurate identification, quantification and monitoring of PQAs with MAM in a single injection (Figure 1).

Key features of an EAD-based MAM workflow

- **Confident PQA identification and differentiation of isomers**: The single-injection EAD platform method allows differentiation of isomers and PTMs that are challenging for traditional characterization and MAM methods
- **Streamlined data flow**: A customizable PQA list connects peptide mapping results in the Biologics Explorer software with PQA quantification and monitoring in a compliance-ready environment in SCIEX OS software
- **Powerful software for peptide mapping**: The Biologics Explorer software offers powerful algorithms and optimized workflows for peptide mapping and PQA selection
- **Compliance-ready**: PQA quantification and monitoring are performed in compliance-ready SCIEX OS software

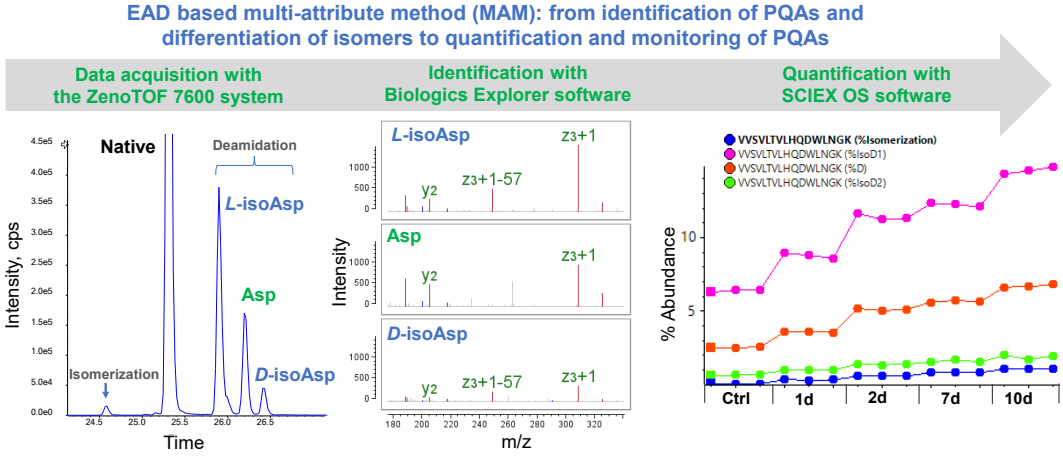

Figure 1. An EAD-based MAM workflow. The EAD platform method[1-4] offered by the ZenoTOF 7600 system provides confident peptide mapping results for sequence confirmation and identification of post-translational modifications (PTMs) and enables differentiation of isomers. This is a prerequisite for achieving accurate quantification results of these PQAs and CQAs for MAM. The combination of the intuitive Biologics Explorer software for peptide mapping and compliance-ready SCIEX OS software for PQA and CQA quantification makes the EAD-based MAM workflow a streamlined, single injection process.

Methods

Sample preparation: The stock solution (10 µg/µL) of NISTmAb (reference material #8671, NIST) was aliquoted into 5 vials. One aliquot (control) was kept frozen and thawed prior to trypsin digestion. The remaining 4 aliquots of NISTmAb were heated at 60ºC for 1 day, 2 days, 7 days or 10 days, respectively. Trypsin digestion of the control and heat-stressed samples was performed following denaturation by guanidine-hydrochloride, reduction with dithiothreitol and alkylation using iodoacetamide. Each sample was analyzed in technical triplicates.

Chromatography: Peptides were separated using an ACQUITY CSH C18 column (2.1 x 150 mm, 1.7 µm, 130 Å, Waters), which was kept at 60ºC in the column oven of an ExionLC system (SCIEX). Table 1 shows the LC gradient used for peptide separation at a flow rate of 0.25 mL/min with mobile phases A and B consisting of 1% formic acid in water and 0.1% FA in acetonitrile, respectively.

Table 1. LC gradients for peptide separation.

Time [min]	A [%]	B [%]
Initial	*99*	*1*
5	*99*	*1*
25	*75*	*25*
65	*60*	*40*
70	*40*	*60*
74	*10*	*90*
74.1	*99*	*1*
75	*99*	*1*
75.1	*10*	*90*
79	*10*	*90*
79.1	*99*	*1*
83	*99*	*1*

Mass Spectrometry: LC-MS data were acquired with an EAD platform method[2-4] in SCIEX OS software using the ZenoTOF 7600 system. The key TOF MS and EAD parameters are listed in Tables 2 and 3, respectively.

Table 2. TOF MS parameters.

Parameter	Values
Ion source gas 1	*50 psi*
Ion source gas 2	*50 psi*
Curtain gas	*35 psi*
CAD gas	*7*
Source temperature	*450ºC*
Spray voltage	*5500 V*
TOF start mass	*200 m/z*
TOF stop mass	*2000 m/z*
Accumulation time	*0.1 s*
Declustering potential	*20 V*
Collision energy	*10 V*
Time bins to sum	*8*

Table 3. EAD parameters.

Parameter	Values
IDA criteria	*Peptide*
Maximum candidate ions	*10*
Intensity threshold	*125 cps*
Charge state	*2 to 10*
Isotope to select	*Most intense*
Exclude time	*6 s after 2 occurrences*
Dynamic ETC for MS/MS	*True*
TOF start mass	*100 m/z*
TOF stop mass	*3000 m/z*
Accumulation time	*0.09 s*
Q1 resolution	*Unit*
Zeno trap	*True*
Electron beam current	*5500 nA*
Electron KE	*7 eV*

Data Processing: Peptide mapping and PQA selection were performed using the “PeptideMapping_Simple” template in the Biologics Explorer software. The PQA list was imported into the Analytics module of the compliance-ready SCIEX OS software for relative quantification and monitoring of PQAs.

Overview of MAM workflow

The MAM workflow described in this technical note takes advantage of the power of the EAD DDA platform method for confident peptide identification and differentiation of isomers, the robust ability of the Biologics Explorer software for peptide mapping and PQA selection and the trusted capability of SCIEX OS software for peak integration, quantification and reporting in a compliance-ready environment. The data flow for this MAM workflow is illustrated in Figure 2. The EAD DDA-based data acquired with the ZenoTOF 7600 system are processed using an optimized peptide mapping workflow template in the Biologics Explorer software. The PQAs confidently identified from peptide mapping are selected and exported to a PQA list (.TXT file), which serves as a connection between the Biologics Explorer software and SCIEX OS software. The PQA list can be directly imported into a processing method within SCIEX OS software. The peaks are then automatically integrated using the well-established MQ4 algorithm. These peak areas are used in the formula function within SCIEX OS software for the calculation of the percent abundances of PQAs. A metric plot can automatically be generated to visualize changes of the percent modification for studies of forced degradation or stability, for example. Finally, a custom report of the results can be generated and exported in different file formats, such as .doc or .pdf.

The isomerization and deamidation peaks of the peptide VVSVLTVLHQDWLNGK (abbreviated as “VVSV peptide”) from the heavy chain of the NISTmAb, HC [305-320], was examined as an example to demonstrate the power of EAD for the differentiation between Asp and isoAsp isomers and the streamlined MAM workflow for the relative quantification of these species.

Confident identification of PQAs using EAD

The EAD platform method is a powerful tool for confident identification and in-depth characterization of a wide range of peptides in a single injection, due to its unique capability for the preservation of labile modifications and differentiation of isomers without the need for optimization.[2-6] Figure 3 highlights the chromatographic separation and identification of the isomerization and deamidation species of the VVSV peptide. While the identification of the isomerization peaks and the localization of deamidation at the Asn residue are straightforward even with CID-based methodologies, the differentiation of the 3 deamidated peaks eluting after the native species poses a challenge for standard MS/MS approaches. The detection of a signature isoAsp fragment (z_3–57) derived from EAD enabled the confident assignment of these 3 deamidated peaks (Figure 3B), 2 of which were present as the isoAsp and 1 as the Asp form. The presence of these 2 isoAsp species can be attributed to aspartic acid racemization, as studied previously[8,9], resulting in

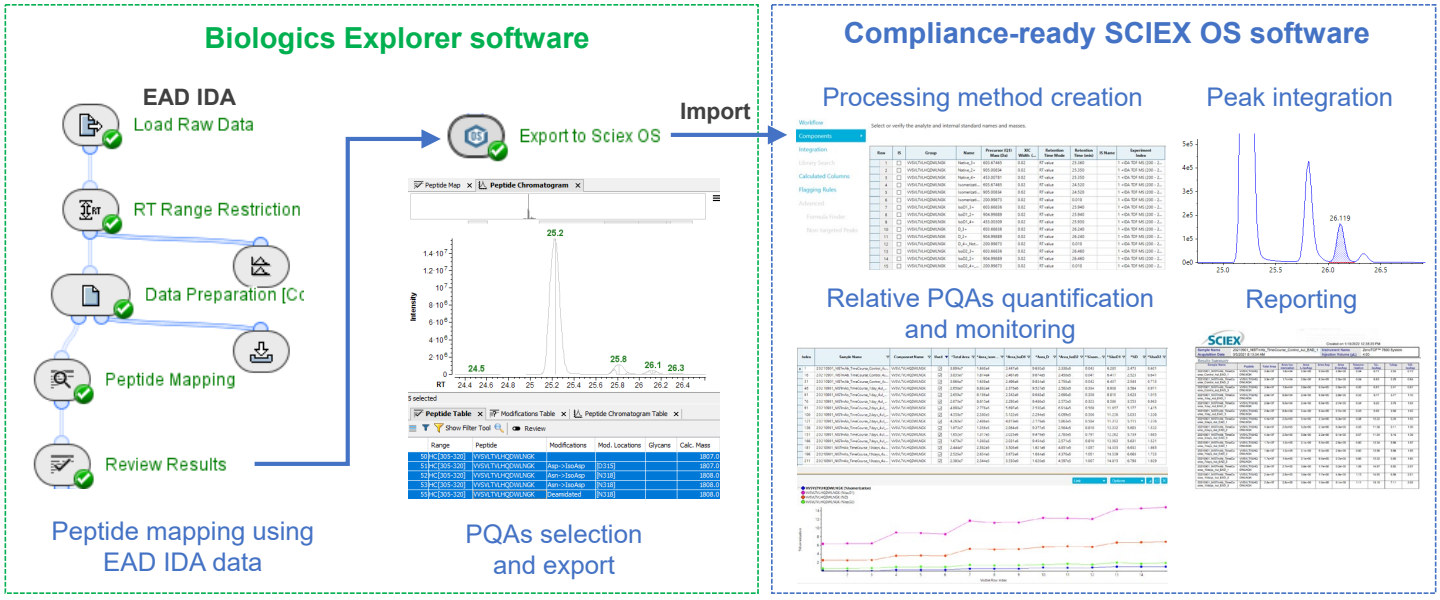

Figure 2. Streamlined MAM workflow from the Biologics Explorer software to compliance-ready SCIEX OS software. In this MAM workflow, the PQAs of a mAb were confidently identified from peptide mapping of EAD DDA data using the Biologics Explorer software. The PQAs of interest are reviewed, selected and exported to a .TXT file, which is then imported into a processing method created using the Analytics module of SCIEX OS software. Peak integration is performed using the MQ4 algorithm, followed by the creation of a custom formula for relative PQA quantification and monitoring. The reporting function offers many options for customization and selection of different file formats, such as .doc and .pdf.

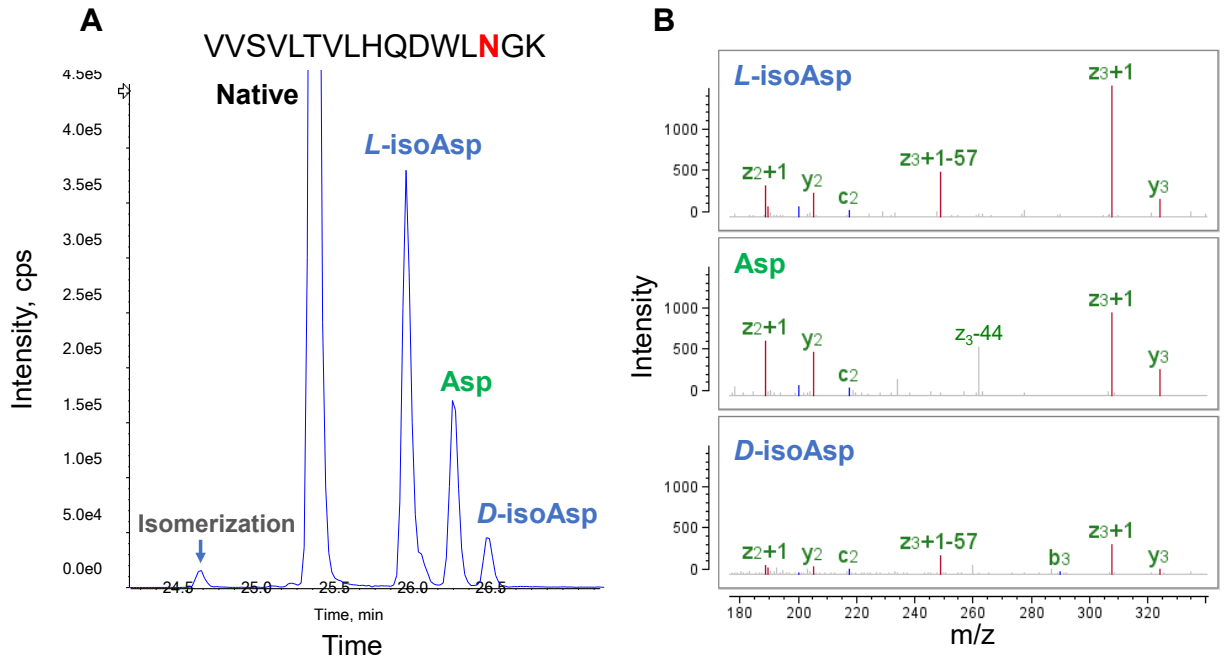

Figure 3. Differentiation between Asp and isoAsp isomers using EAD. The native, isomerization and deamidation species of the VVSV peptide were chromatographically separated (A) and confidently identified by MS/MS using EAD (B). The detection of signature fragments[5,7] (z_3–57 and z_3–44) (B) allowed differentiation between the Asp and isoAsp isomers, leading to unambiguous assignment of the 3 peaks eluting after the native species. The 2 isoAsp peaks may be attributed to aspartic acid racemization[7,8] and can be assigned as the *L*- and *D*- forms, respectively.

partial conversion of the *L*-form (*L*-isoAsp) to its racemic D-counterpart (*D*-isoAsp). These results demonstrate the importance of using EAD for accurate identification of PQAs.

Selection and export of PQAs

The Biologics Explorer software offers intuitive control and powerful visualization for reviewing results and selecting PQAs. As illustrated in Figure 4, the isomerization and deamidation peaks of the VVSV peptide were selected in the peptide table, with their XICs displayed in the peptide chromatogram tab above the table. The PQAs selected were exported into a .TXT file using the “Export to Sciex OS” node in the Biologics Explorer software. Subsequently, this file was imported for creating the quantification method.

Peak integration, relative quantification and monitoring of PQAs

In the MAM workflow, the creation of the processing method, peak integration and relative quantification of PQAs were all performed using the Analytics module within SCIEX OS software (Figure 2).

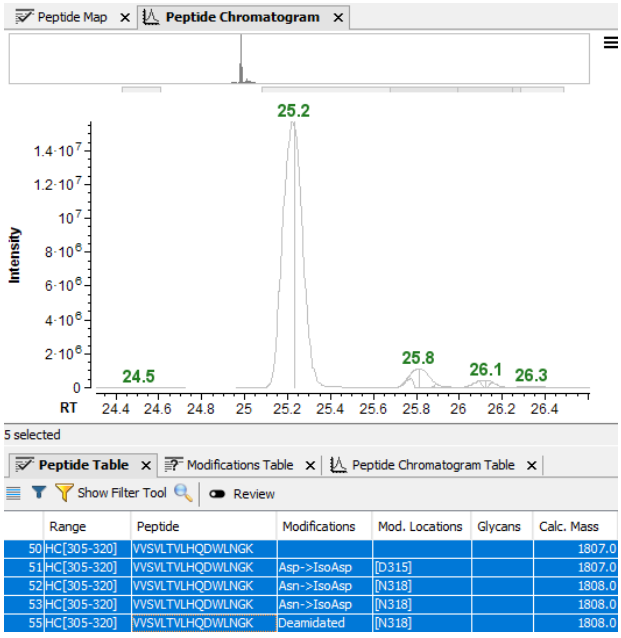

Figure 4. PQA selection in the Biologics Explorer software. The PQAs are selected in the peptide table, while their XICs can be viewed in the peptide chromatogram tab above the table.

The Analytics module offers the powerful and well-established MQ4 algorithm for reproducible peak integration with minimal optimization. A comprehensive set of adjustable parameters is accessible to advanced users who may want to fine-tune the integration for certain applications. In this study, each isomer of the deamidated VVSV peptide was properly integrated with the defined quantification method (Figure 5), leading to accurate quantification of these species.

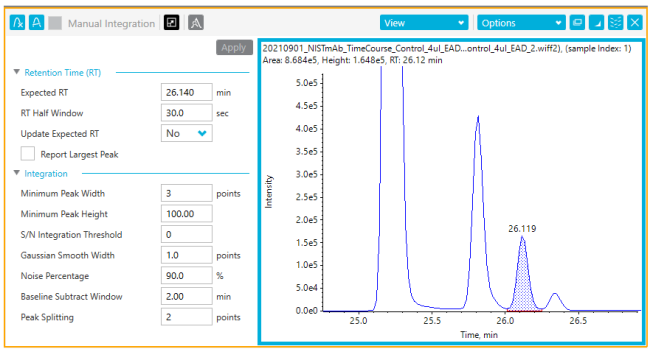

Figure 5. Peak integration in SCIEX OS software. The Asp isomer of VVSV peptide at RT = 26.12 min was accurately integrated using the MQ4 algorithm.

The Analytics module provides full flexibility to build custom formulas in the processing method to calculate the percent abundances of PQAs.[10-11] Figure 6 displays the quantification results of the PQAs of the VVSV peptide, obtained by applying the processing method with a custom formula to 3 replicate injections of the NISTmAb control and heat-stressed samples. The calculated percent abundances were highly reproducible within replicates for both CID and EAD methods, allowing for quantitative rigor (%CV <10%, see Table 4).

These percent values increased notably for the Asp and different isoAsp forms, as the duration of heat stress applied increased from 0 to 10 days. To quickly examine results, SCIEX OS software offers metric plots for visualization (Figure 7A). Alternatively, the results can be exported and processed in Excel using other plot options (Figure 7B).

In summary, the results described in this technical note highlight the power of using the EAD platform method for confident identification of PQAs and a streamlined data flow between the Biologics Explorer software and the compliance-ready SCIEX OS software for the implementation of a comprehensive single injection MAM workflow.

Index	Sample Name	Component Name	Used	*Total Area	*Area_Iso...	*Area_IsoD1	*Area_D	*Area_IsoD2	*%Isome...	*%IsoD1	*%D	*%IsoD2
1	Control	VVSVLTVLHQDWLNGK	☑	3.786e7	1.614e4	2.540e6	9.561e5	2.777e5	0.043	6.708	2.526	0.734
16		VVSVLTVLHQDWLNGK	☑	3.901e7	1.689e4	2.587e6	8.919e5	2.512e5	0.043	6.630	2.286	0.644
31		VVSVLTVLHQDWLNGK	☑	3.878e7	1.761e4	2.564e6	9.576e5	2.609e5	0.045	6.613	2.469	0.673
46	1 Day	VVSVLTVLHQDWLNGK	☑	2.585e7	8.616e4	2.370e6	9.755e5	2.844e5	0.333	9.167	3.773	1.100
61		VVSVLTVLHQDWLNGK	☑	2.611e7	8.987e4	2.415e6	9.810e5	2.722e5	0.344	9.249	3.757	1.042
76		VVSVLTVLHQDWLNGK	☑	2.646e7	8.771e4	2.396e6	9.390e5	2.703e5	0.332	9.054	3.549	1.022
91	2 Days	VVSVLTVLHQDWLNGK	☑	4.386e7	2.532e5	5.358e6	2.301e6	6.288e5	0.577	12.218	5.247	1.434
106		VVSVLTVLHQDWLNGK	☑	4.516e7	2.484e5	5.229e6	2.305e6	6.262e5	0.550	11.580	5.105	1.387
121		VVSVLTVLHQDWLNGK	☑	4.362e7	2.482e5	5.035e6	2.249e6	6.060e5	0.569	11.544	5.157	1.389
136	7 Days	VVSVLTVLHQDWLNGK	☑	1.673e7	1.344e5	2.064e6	9.469e5	2.627e5	0.803	12.340	5.660	1.571
151		VVSVLTVLHQDWLNGK	☑	1.647e7	1.320e5	2.068e6	9.318e5	2.556e5	0.802	12.558	5.658	1.552
166		VVSVLTVLHQDWLNGK	☑	1.652e7	1.401e5	2.067e6	9.621e5	2.732e5	0.848	12.515	5.825	1.654
181	10 Days	VVSVLTVLHQDWLNGK	☑	2.533e7	2.748e5	3.766e6	1.729e6	5.234e5	1.085	14.865	6.824	2.066
196		VVSVLTVLHQDWLNGK	☑	2.449e7	2.756e5	3.638e6	1.684e6	4.929e5	1.125	14.854	6.875	2.013
211		VVSVLTVLHQDWLNGK	☑	2.487e7	2.762e5	3.776e6	1.767e6	5.098e5	1.111	15.187	7.107	2.050

Figure 6. Results table of relative quantification of PQAs in the Analytics module of SCIEX OS software. The table lists the peak areas and percent abundances of the isomerization and deamidation peaks of the VVSV peptide across 3 replicate injections of the NISTmAb control and heat-stressed samples.

Table 4. Summary of CVs measured for isomerization and deamidation species of the VVSV peptide by CID and EAD methods.

	Isomerization		*L*-isoAsp		Asp		*D*-isoAsp	
	CID	EAD	CID	EAD	CID	EAD	CID	EAD
Control	6.01	2.64	1.41	0.76	1.45	5.17	8.71	6.72
1 Day	4.06	1.98	2.03	1.07	1.05	3.38	2.85	3.84
2 Day	1.72	2.45	1.97	3.22	1.41	1.39	2.80	1.89
7 Day	0.93	3.21	1.00	0.93	1.27	1.68	5.60	3.41
10 Day	0.76	1.83	1.66	1.26	1.31	2.17	7.03	1.33

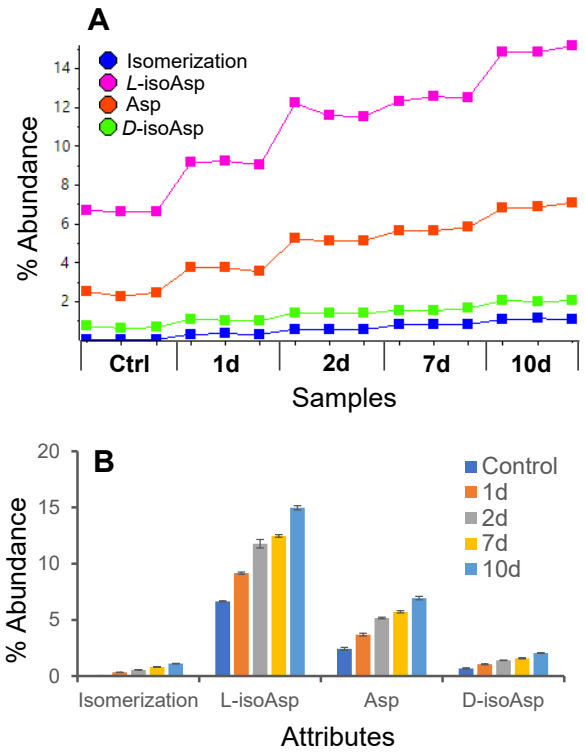

Figure 7. Visualization of the MAM results. The metric plot (A) and bar chart (B) both showcase the reproducibility of PQA quantification for replicate injections and the ability of the MAM workflow to monitor the percent abundances of PQAs for a time-course study.

Conclusions

- Confident identification of PQAs and unambiguous differentiation of their isomers are critical for the successful implementation of a MAM
- Differentiation between Asp and isoAsp isomers from peptide deamidation, an essential class of PQAs that may affect a drug's efficacy and safety, was achieved with a DDA platform method using EAD
- Efficient identification was enabled in the Biologics Explorer software through ready-to-use workflow templates that provided confident peptide mapping results and visualization tools with the ability to export a PQA list to SCIEX OS software for quantification
- The compliance-ready SCIEX OS software offered robust algorithms and tools for peak integration and relative quantification of PQAs
- An effective MAM workflow from identification to quantification was achieved by data flow between the Biologics Explorer software and compliance-ready SCIEX OS software

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