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Demonstration of an improved multi-attribute methodology (MAM) using alternative fragmentation to enhance the identification of product quality attributes (PQAs)

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ABSTRACT

Here, the power of a data-dependent acquisition (DDA) platform method for the identification of product quality attributes (PQAs) of a monoclonal antibody (mAb) and their quantification and monitoring using alternative fragmentation is demonstrated. The streamlined multi-attribute method (MAM) workflow allows for superior identification and quantification in a single injection.

INTRODUCTION

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 was employed as the first step for identification, followed by quantification with either only MS or combined 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[1], which enables confident and accurate identification, quantification and monitoring of PQAs with MAM in a single injection, increasing efficiency and throughput.

MATERIALS AND 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.

Table 1. Chromatographic conditions.

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*

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.

Mass spectrometry: LC-MS data were acquired with an EAD platform method[1] in SCIEX OS software using the ZenoTOF 7600 system. The key TOF MS and EAD parameters are listed in Tables 2 and 3, respectively.

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.

Table 3. TOF MS parameters.

Parameter	Values
Ion source gas 1	*50 psi*
Ion source gas 2	*50 psi*
Curtain gas	*35 psi*
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*

Table 3. EAD parameters.

Parameter	Values
Maximum candidate ions	*10*
Charge state	*2 to 10*
Exclude time	*6 s after 2 occurrences*
TOF start mass	*100 m/z*
TOF stop mass	*3000 m/z*
Accumulation time	*0.09 s*
Zeno trap	*True*
Electron beam current	*5500 nA*
Electron KE	*7 eV*

RESULTS

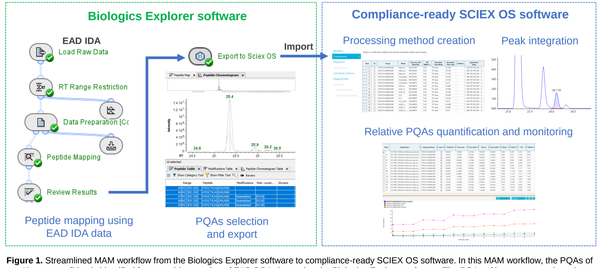

Figure 1. Streamlined MAM workflow from the Biologics Explorer software to compliance-ready SCIEX OS software. In this MAM workflow, the PQAs of a mAb were confidently identified from peptide mapping of EAD DDA data using the Biologics Explorer software. The PQAs of interest are reviewed, selected and 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.

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. 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 (Figure 1).

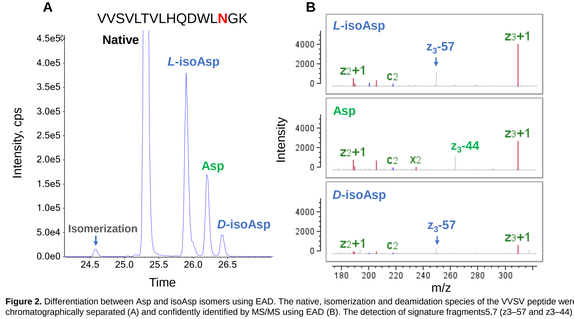

Figure 2. Differentiation between Asp and isoAsp isomers using EAD. The native, isomerization and deamidation species of the VVSV peptide were chromatographically separated (A) and confidently identified by MS/MS using EAD (B). The detection of signature fragments5,7 (z_3–57 and z_3–44) (B) allowed differentiation 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[2,3] and can be assigned as the L- and D- forms, respectively.

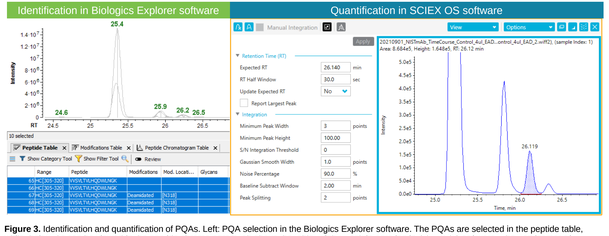

Figure 3. Identification and quantification of PQAs. Left: PQA selection in the Biologics Explorer software. The PQAs are selected in the peptide table, while their XICs can be viewed in the peptide chromatogram tab above the table. Right: Peak integration in SCIEX OS software. The Asp isomer of VVSV peptide at RT = 26.12 min was accurately integrated using the MQ4 algorithm.

While the identification of the isomerization peak and the localization of deamidation at the Asn residue are straightforward even with CID-based methods, the differentiation of the 3 deamidated peaks eluting after the native species poses a challenge (Figure 2A). The detection of a signature isoAsp fragment (z_3–57) derived from EAD enabled the confident assignment of these 3 deamidated peaks (Figure 2B), 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[2,3], resulting in 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.

The percent values increased notably for the Asp and different isoAsp forms, as the duration of heat stress increased from 0 to 10 days (Figure 4 and 5).

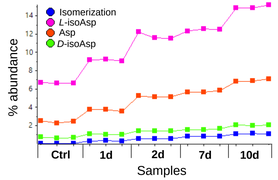

Figure 4. Visualization of the MAM results. The metric plot showcases the reproducibility of PQA quantification for replicate injections (n = 3) and the ability of the MAM workflow to monitor the percent abundances of PQAs for a time-course study.

Figure 5 displays the quantification results of the PQAs of the VVSV peptide, obtained by applying the processing method with a custom formula for %abundance calculation. The custom formulas allow for full quantitative flexibility, such as summing of charge states or quantification of monoisotope vs other isotopes. The calculated %abundances obtained from the stress study were highly reproducible within replicates (n = 3) using the EAD DDA method. The tight %CVs <10% (Table 4), even in case of very low-abundance species (<1% relative abundance), allow for confident quantification of all monitored species.

In summary, the fast EAD DDA method was proven to enable in-depth characterization of challenging species and quantification within 1 injection without the need for optimization of EAD parameters. By combining quantitative monitoring with a highly reproducible alternative fragmentation DDA approach for identification purposes, efficiency can be significantly increased. Furthermore, unexpected unknown features can be identified without the need for rerunning samples.

Table 4. Summary of %CVs for deamidation species of the VVSV peptide using EAD DDA.

Parameter	Isomerization	*L*-isoAsp	Asp	*D*-isoAsp
Control	*2.64*	*0.76*	*5.17*	*6.72*
1 day	*1.98*	*1.07*	*3.38*	*3.84*
2 days	*2.45*	*3.22*	*1.39*	*1.89*
7 days	*3.21*	*0.93*	*1.68*	*3.41*
10 days	*1.83*	*1.26*	*2.17*	*1.33*

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	1.614e6	2.540e6	9.561e5	2.777e5	0.043	6.708	2.526	0.734
16			☑	3.901e7	1.689e6	2.587e6	8.919e5	2.512e5	0.043	6.630	2.286	0.644
31			☑	3.878e7	1.761e6	2.564e6	9.576e5	2.609e5	0.045	6.613	2.469	0.673
46	1 day	VVSVLTVLHQDWLNGK	☑	2.585e7	8.616e6	2.370e6	9.755e5	2.844e5	0.333	9.167	3.773	1.100
61			☑	2.611e7	8.987e6	2.415e6	9.810e5	2.722e5	0.344	9.249	3.757	1.042
76			☑	2.646e7	8.771e6	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			☑	4.516e7	2.484e5	5.229e6	2.305e6	6.262e5	0.550	11.580	5.105	1.387
121			☑	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			☑	1.647e7	1.320e5	2.068e6	9.318e5	2.556e5	0.802	12.558	5.658	1.552
166			☑	1.652e7	1.401e5	2.067e6	9.621e5	2.732e5	0.848	12.515	5.825	1.654
181	10 days	VVSVLTVLHQDWLNGK	☑	2.513e7	2.748e5	3.765e6	1.729e6	5.234e5	1.085	14.865	6.824	2.066
196			☑	2.449e7	2.756e5	3.638e6	1.684e6	4.929e5	1.125	14.854	6.875	2.013
211			☑	2.487e7	2.762e5	3.776e6	1.767e6	5.098e5	1.111	15.187	7.107	2.050

Figure 5. 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.

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 accurate state-of-the-art 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 with superior information gain with Zeno EAD in the ZenoTOF 7600 system, Biologics Explorer software and SCIEX OS software

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