Monitoring and determining the cause of antibody discoloration using capillary isoelectric focusing (cIEF) and electron activated dissociation (EAD)

Featuring the ZenoTOF 7600 system, BioPhase 8800 system, Biologics Explorer software and an EAD-based multiple attribute methodology (MAM) workflow from SCIEX

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This technical note highlights a streamlined solution that combines the power of cIEF to monitor antibody discoloration and an EAD-based MAM workflow to determine the cause of color change. The cIEF profiles of the time-course forced glycation samples of NISTmAb revealed a significant increase in acidic variants in the discolored samples. EAD-based MAM was employed to identify and quantify the acidic species to assess their role in the discoloration of protein therapeutics.

Color is a potential critical quality attribute of protein therapeutics.[1] Undesirable color changes can lead to increased product heterogeneity and concerns about the safety and efficacy of a drug. Hence, the cause of color change in biotherapeutics must be fully understood and reported to regulatory agencies. Previous studies showed that advanced glycation end products (AGEs) are partially responsible for the discoloration of recombinant antibodies.[2,3] AGEs are often challenging to characterize using collision-based MS/MS approaches because they are ineffective for fragmenting these species.[3-5] In contrast, EAD-based MAM enabled confident identification and relative quantification of the glycated species and AGEs, providing molecular insights into the role of AGEs in the discoloration of protein therapeutics.[3-6] Here, the cIEF and EAD-based MAM workflows were leveraged together (Figure 1) to provide a streamlined solution from product quality tracking to cause investigation.

Key features of cIEF and EAD-based MAM workflows for product quality assessment

- **Streamlined method:** cIEF and EAD-based MAM workflows offer an integrated solution to monitor and provide molecular insights into product changes such as discoloration
- **Comprehensive characterization:** EAD provides excellent fragmentation of the peptide backbone and accurate localization of labile modifications
- **Quantitatively reproducible:** EAD-based MAM offers reproducible quantification results for product quality attributes

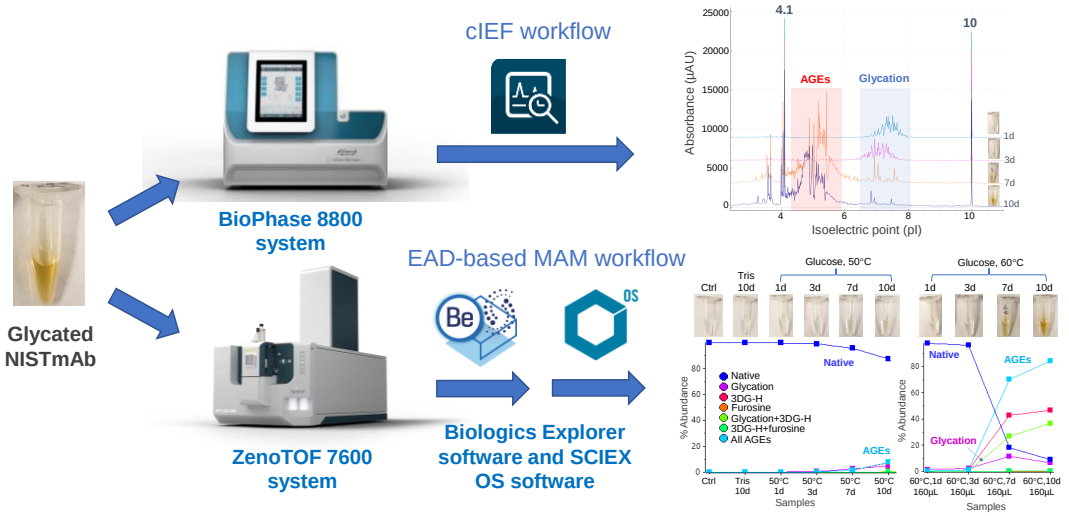

Figure 1. Characterization of the discolored NISTmAb using the cIEF and EAD-based MAM workflows. The cIEF results obtained from the BioPhase 8800 system revealed a gradual increase in acidic variants in the time-course forced degradation samples of NISTmAb without color change but a dramatic increase in acidic species in the discolored samples. Relative quantification of glycation and AGEs by the EAD-based MAM workflow established a correlation between the color change and the level of AGEs but not glycated species present, consistent with the cIEF data.

Methods

Sample preparation: The non-stressed control sample of NISTmAb (RM 8671, NIST) was diluted to 5 µg/µL in 25mM Tris-HCl (pH=7.4) buffer and kept at -40°C for 10 days prior to enzymatic digestion. The stressed samples were prepared by incubating 5 µg/µL of NISTmAb in 25mM Tris-HCl (pH=7.4) at 60°C for 7-10 days or in 500mM glucose (Sigma-Aldrich) at 50°C or 60°C for up to 10 days. While 200 µL of NISTmAb samples were incubated at 50°C, two different volumes of the samples (160 µL and 40 µL) were tested for incubation at 60°C. The time-course glycation samples were split into 2 portions for subsequent LC-MS and cIEF analyses.

The samples for LC-MS analysis were denatured in guanidine hydrochloride, reduced with dithiothreitol and alkylated with iodoacetamide. Buffer exchange was then performed using Bio-Spin 6 columns (Bio-Rad Laboratories) and the samples were digested using chymotrypsin or Glu-C (Promega). The final digests were injected in 20 µL aliquots (~10 µg) for EAD analysis.

Chromatography: The 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 the peptide separation. The column was kept at 60°C in the column oven of an ExionLC system (SCIEX). Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile.

cIEF separation: Buffers and reagents were prepared following the instructions in the cIEF kit for BioPhase 8800 system application guide.[7] The cIEF master mix was prepared according to the optimized conditions published in the literature.[8] The cIEF data were acquired using the BioPhase 8800 system (SCIEX). For cIEF separation, the focusing and mobilization steps were performed using 25 kV for 20 min and 30 kV for 30 min, respectively. The UV detection wavelength was 280 nm.

Table 1. LC gradient for peptide separation.

Time (min)	A (%)	B (%)
Initial	*98*	*2*
2	*98*	*2*
62	*65*	*35*
65	*50*	*50*
67	*10*	*90*
70	*10*	*90*
71	*98*	*2*
75	*98*	*2*

Mass spectrometry: EAD data-dependent acquisition (DDA) experiments were performed using the ZenoTOF 7600 system and SCIEX OS software. The key TOF MS and MS/MS settings used are listed in Tables 2 and 3, respectively.

Data processing: EAD data were analyzed using the peptide mapping workflow templates in the Biologics Explorer software, as described in previous technical notes.[4-6]

Table 2. TOF MS parameters.

Parameter	Value
Spray voltage	*5500 V*
TOF start mass	*400 m/z*
TOF stop mass	*1800 m/z*
Accumulation time	*0.1 s*
Source temperature	*400°C*
Declustering potential	*80 V*
Collision energy	*10 V*
Time bins to sum	*8*

Table 3. MS/MS parameters using EAD.

Parameter	EAD
IDA criteria	*Peptide*
Maximum candidate ions	*10*
Charge state	*2-10*
Isotope to select	*Most intense*
Exclude time	*4s after 2 occurrences*
Dynamic ETC for MS/MS	*True*
Start mass	*100 m/z*
Stop mass	*2,000 m/z*
Zeno trap	*ON*
Zeno threshold	*100,000 cps*
Accumulation time	*0.1 s*
Declustering potential	*80 V*
Time bins to sum	*10*
Electron beam current	*5,500 nA*
Electron KE (eV)	*7 eV*
Reaction time	*20 ms*

Discoloration of protein therapeutics

Previous studies have shown that recombinant antibodies produced from Chinese hamster ovary cells can show visible yellow or brown color.[1-3] The discoloration of antibody-based therapeutics indicates the presence of fluorescent impurities, which can affect the safety and efficacy of a drug product.[1-3] Therefore, it is critical to understand the cause of discoloration on the molecular level to fully assess the effect of color change on product quality.

It was demonstrated previously that the degree of AGE modification is a better indicator of color change than oxidation and glycation.[2] This notion was supported by a correlation between the discoloration and the relative level of AGEs measured using the EAD-based workflow.[6] In this work, the factors that affect the discoloration of NISTmAb were further investigated. The powerful capabilities of cIEF and EAD-based MAM workflows were leveraged to monitor the charge profile of NISTmAb and determine the cause of discoloration at the molecular level.

Figure 2 shows the color of different volumes (40 µL, 160 µL or 200 µL) of 5 µg/µL NISTmAb solutions incubated at different temperatures (50°C or 60°C). The 200 µL samples stressed at 50°C with or without glucose did not undergo visible color change during the 10 days of incubation (Figure 1A). In contrast, the discoloration became visible in the 160 µL samples incubated at 60°C by day 7 (Figure 1B), indicating that the color change was accelerated at higher temperatures. The onset of the color change was expedited when NISTmAb was incubated with glucose in a smaller volume of 40 µL. As displayed in Figure 1C, the yellow or brown color was visible in the 40 µL sample incubated at 60°C by day 3 and the color grew darker by day 6. These results indicate that the discoloration of protein therapeutics can be affected by factors including the presence of glucose, temperature, duration of incubation and sample volume.

Monitoring charge profiles of glycated NISTmAb by cIEF

It was demonstrated previously that the BioPhase 8800 system enabled the reproducible separation of protein therapeutics in either the non-reduced or reduced form.[9] The cIEF technology employed by the BioPhase 8800 system enables rapid assessment and monitoring in stability or forced degradation studies of protein therapeutics. In this work, the BioPhase 8800 system was used to track the charge profiles of NISTmAb in the time-course forced glycation samples, including those displaying color change. The changes detected by cIEF were further elucidated using an EAD-based MAM workflow.

The aligned cIEF electropherograms of the control and stressed samples of NISTmAb are shown in Figure 3. Compared to the control sample, a significant increase in the number of acidic variants was detected in the NISTmAb solutions incubated at 50°C in the presence of glucose (Figure 3A). The charge profiles shifted gradually to lower pI regions while the profile distribution became broader with the increasing incubation duration. For instance, the charge profile of NISTmAb was centered at pI 8.0-8.5 when incubated at 50°C for 1 day. When the sample was incubated at 50°C for 10 days, however, a broader distribution of peaks was detected in the pI range of 6-7 (Figure 3A). This pI shift was also observed between the samples incubated at different temperatures. A pI range of 8.0-8.5 was observed when the sample was incubated at 50°C for 1 day (Figure 3A), whereas a pI range of 7-8 was observed when the sample was incubated at 60°C for 1 day (Figure 3B). A drastic change in the charge profile was observed in the 2 samples incubated at 60°C that underwent color change at day 7 and day 10 (Figure 3B). In these samples, the acidic species were mostly detected in the pI range of 4-6, whereas these species were observed at pI 6-9 in the samples without discoloration.

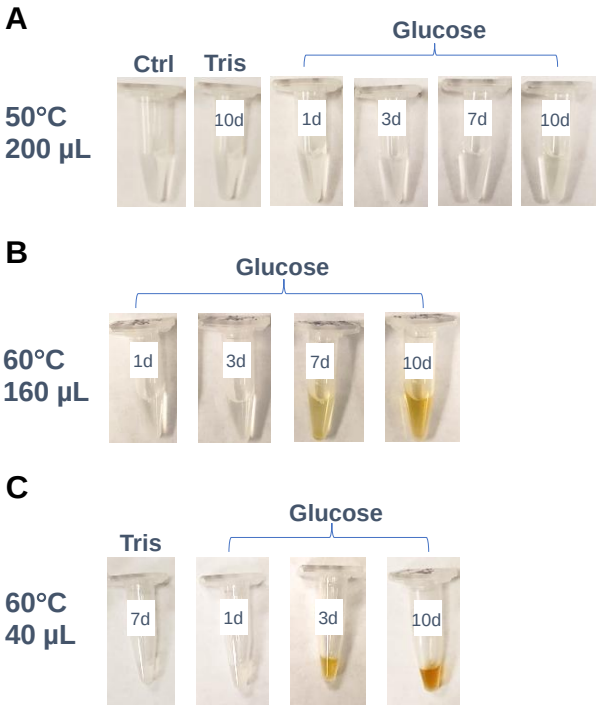

Figure 2. Time-course forced glycation of NISTmAb. The control (Ctrl in A) and NISTmAb samples that were thermally stressed in the presence of Tris-HCl only (Tris in A and C) did not display color change. No discoloration was observed for the NISTmAb samples that were incubated with glucose at 50°C (samples 1d-10d in A). By comparison, a visible color change was detected in the samples that were incubated at 60°C for an extended period of time (7 days in B and 3 days in C). The color change occurred more quickly in samples that had a smaller incubation volume (40 µL in C vs. 160 µL in B).

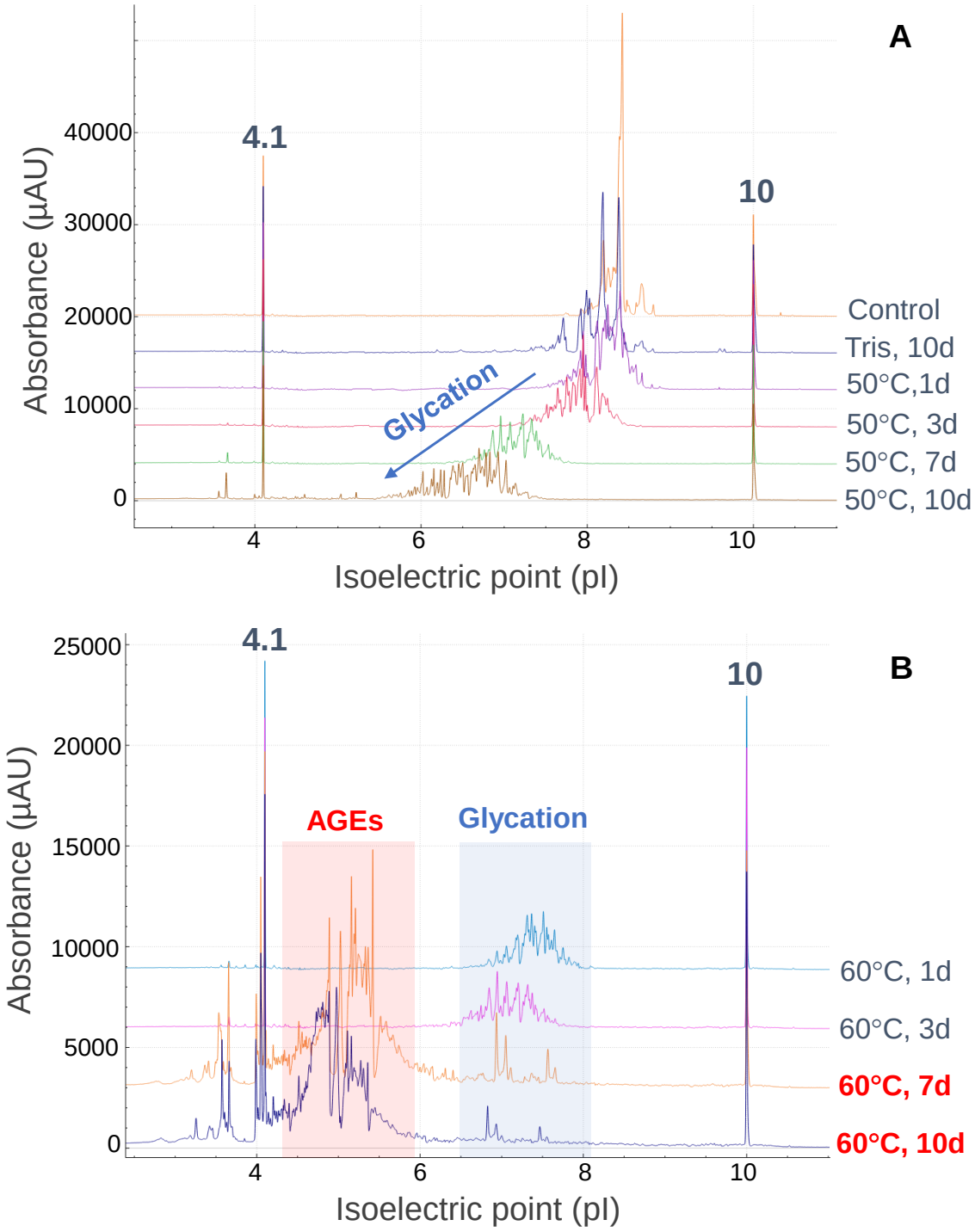

Figure 3. The overlaid cIEF electropherograms of time-course forced glycation samples of NISTmAb. The peaks at pI 4.1 and pI 10 correspond to pI markers used in cIEF experiments. In the 50°C samples that did not undergo color change (A), the cIEF data revealed a gradual shift of the charge profile of NISTmAb to the acidic region (lower pI) with increasing incubation duration. This gradual pI shift can be attributed to the increasing level of the glycated species. A dramatic shift of the charge profile was observed in the 2 discolored samples that were incubated at 60°C (highlighted in red in B), in which nearly all the acidic species were detected at pI <6, compared to pI 6-9 in the samples without color change. LC-MS analyses identified the AGEs as the species that were likely responsible for the significant pI shift and discoloration.

The results demonstrate the power of cIEF for the rapid assessment of product changes, such as discoloration, associated with the forced degradation of protein therapeutics. EAD-based MAM was next applied to complement cIEF and understand the molecular cause of the discoloration.

EAD-based MAM for identification and quantification of glycation and AGEs

The EAD-based MAM workflow provides a streamlined approach for the identification and relative quantification of product quality attributes (PQAs), including challenging modifications such as glycation and AGEs.[6] This workflow encompasses the impactful capabilities of EAD technology for the comprehensive characterization of protein therapeutics, the easy-to-use Biologics Explorer software for PQA identification and the industry-leading algorithms of SCIEX OS software for PQA quantification.

The characterization capabilities of EAD for the glycated and AGE species have been showcased in previous technical notes.[4-6] The deisotoped EAD MS/MS spectra of 2 selected AGEs from Biologics Explorer software are displayed in Figure 4. These 2 AGEs identified from the trypsin/Lys-C and Glu-C digests contained a 3-deoxyglucosone hydroimidazolone (3DG-H) modification on the same arginine (Arg) residue near the N-terminus of the heavy chain. EAD led to a complete

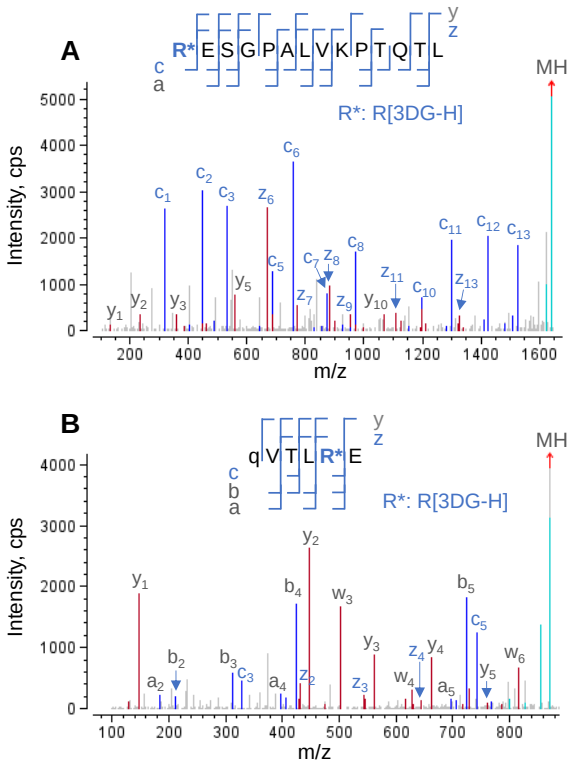

Figure 4. Deisotoped EAD MS/MS spectra of 2 AGEs derived from the same Arg residue. EAD led to excellent fragmentation of 2 AGEs containing the 3DG-H modification on the same Arg residue in 2 peptides from the trypsin/Lys-C (A) and Glu-C (B) digestions, respectively. q = pyroglutamic acid.

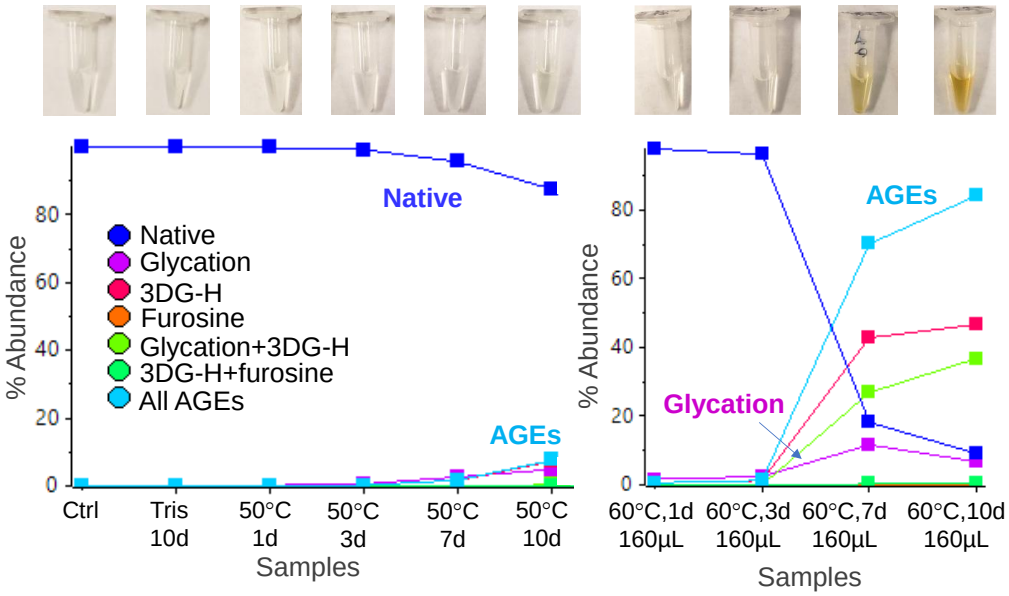

Figure 5. Relative quantification of glycation and AGEs using the EAD-based MAM workflow. Relative abundances of the native, glycated and AGE species of the peptide RESGPALVKPTQTL were calculated and plotted using the Analytics module in SCIEX OS software. The color of each sample is shown at the top of the plots to facilitate comparison. The AGEs line corresponds to the sum of the relative abundances of all AGE species identified. These data revealed a correlation between discoloration and the relative abundance of AGEs but not the relative abundance of glycation.

*Samples	*%Native...	*%Hex_RESG	*%3DGH_RESG	*%Furosine...	*%Hex_3DGH...	*%3DGH_Furosine ...	*%AGEs
Control	99.94	0.06	0.00	0.00	0.00	0.00	0.000
Tris-HCl, 10d	99.97	0.03	0.00	0.00	0.00	0.00	0.000
Glucose, 50c, 1d	99.73	0.22	0.05	0.00	0.00	0.00	0.054
Glucose, 50c, 3d	99.17	0.61	0.22	0.00	0.00	0.00	0.216
Glucose, 50c, 7d	95.64	2.65	1.71	0.00	0.00	0.00	1.711
Glucose, 50c, 10d	87.52	4.88	7.26	0.00	0.34	0.00	7.605
Glucose, 60c, 1d	97.71	1.67	0.62	0.00	0.00	0.00	0.619
Glucose, 60c, 3d	96.24	2.44	1.29	0.00	0.03	0.00	1.320
Glucose, 60c, 7d	18.21	11.67	42.81	0.19	26.84	0.28	70.122
Glucose, 60c, 10d	9.10	6.75	46.63	0.17	36.79	0.55	84.146

Figure 6. The results table from SCIEX OS software that shows the relative abundances of glycation and AGEs identified for the peptide RESGPALVKPTQTL in the control and time-course samples. The native peptide was the dominant species (>96%) detected in the 4 samples that did not develop visible color. These samples included the control, the day 10 Tris-HCl stress sample and the day 1 and day 3 glucose stress samples. The level of glycation increased in the first 7 days of incubation with glucose but then decreased by day 10. In comparison, the relative abundances of AGEs containing 3DG-H increased significantly by day 7 and remained the dominant species in the day 10 sample.

fragmentation of these 2 AGEs, enabling confident identification and localization of the AGE moiety.

Relative quantification of glycated and AGE species in the tryptic peptide RESGPALVKPTQTL was performed using the Analytics module within SCIEX OS software. The quantification results were visualized using a metric plot (Figure 5) and a results table (Figure 6). The results showed that the relative abundances of the AGE modifications were significantly increased in the 2 discolored samples compared to the samples that did not undergo color change. For example, the percent abundance of all AGEs for the peptide RESGPALVKPTQTL was ~1.3% in the 160 µL sample that was incubated at 60°C for 3 days, whereas the abundance of AGEs was ~70.1% in the sample that was incubated at 60°C for 7 days (Figures 5 and 6). The LC-MS results indicated that AGEs were the main species observed at pI<6 in the cIEF data of the 2 discolored samples (Figure 3), while glycated species contribute more to peaks at pI 6-8. Together, the LC-MS and cIEF data indicated that the AGEs are partially responsible for the discoloration of antibody-based therapeutics.[2]

In summary, the data described in this technical note provide additional insight into the factors that govern the discoloration of protein therapeutics and the role of AGEs in color change. Additional studies will be necessary to determine the specific AGEs that might produce the characteristic yellow or brown color observed.

Conclusions

- The streamlined cIEF and EAD-based MAM workflows offer a useful solution to monitor the changes in charge profile associated with antibody discoloration and determine the cause of color change
- The color change of NISTmAb was affected by factors including the absence or presence of glucose, temperature, duration of incubation and sample volume
- The cIEF data revealed a shift of the charge profile to pI<6 in the samples that displayed the distinct yellow or brown color
- EAD-based MAM identified AGEs as the species that is likely responsible for the discoloration of antibody-based therapeutics

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