Elucidation and tracking of charge variants in antibodies: combining ease-of-use with a gain in information

Cation exchange chromatography (CEX) separation with the SCIEX X500B QTOF LC-MS/MS System and SCIEX OS Software 1.7 for intact multiple attribute methodology (intact MAM)

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A variety of modifications can lead to differences in the surface charge of antibodies such as sialylation, deamidation, oxidation, glycation, C-terminal lysine, etc. In the case of protein therapeutics, the charge heterogeneity profile needs to be characterized and monitored as it has a potential impact on a product's safety and/or efficacy. CEX has been widely adopted to obtain this information in development, but also in production and lot release.[1,2] However, conventional CEX methods utilize non-volatile buffer systems, which are not compatible with mass spectrometry (MS). Lengthy offline sample collection and preparation was required to clarify peak identity by subsequent MS-based detection. With recent advances, coupling CEX separation with high resolution MS can be achieved, allowing for separation and identification of charged variants simultaneously.[3] Nevertheless, monitoring complex charge variant profiles across multiple samples in an easy fashion remains a challenge. The emergence of multiple attribute methodologies (MAM) including software approaches, especially based on intact level, can be a solution.

Here, a liability study focusing on the charge heterogeneity profile (Figure 1) via CEX in combination with the SCIEX X500B QTOF System is demonstrated. The SCIEX flexible solution for MAM within SCIEX OS Software 1.7 is employed to track the changes utilizing intact reconstructed data for each charge variant.

Key features of SCIEX MAM solution for CEX based charge heterogeneity analysis

- Fast and reproducible CEX method with MS-friendly, volatile mobile phase system enabling an excellent separation of different charged isoforms
- High-quality native-like MS data allowing for an additional dimension of information to clarify the identity of charge variants
- Complete software solution for acquisition and quantitative tracking of attribute changes in an intuitive, yet, easy to use fashion

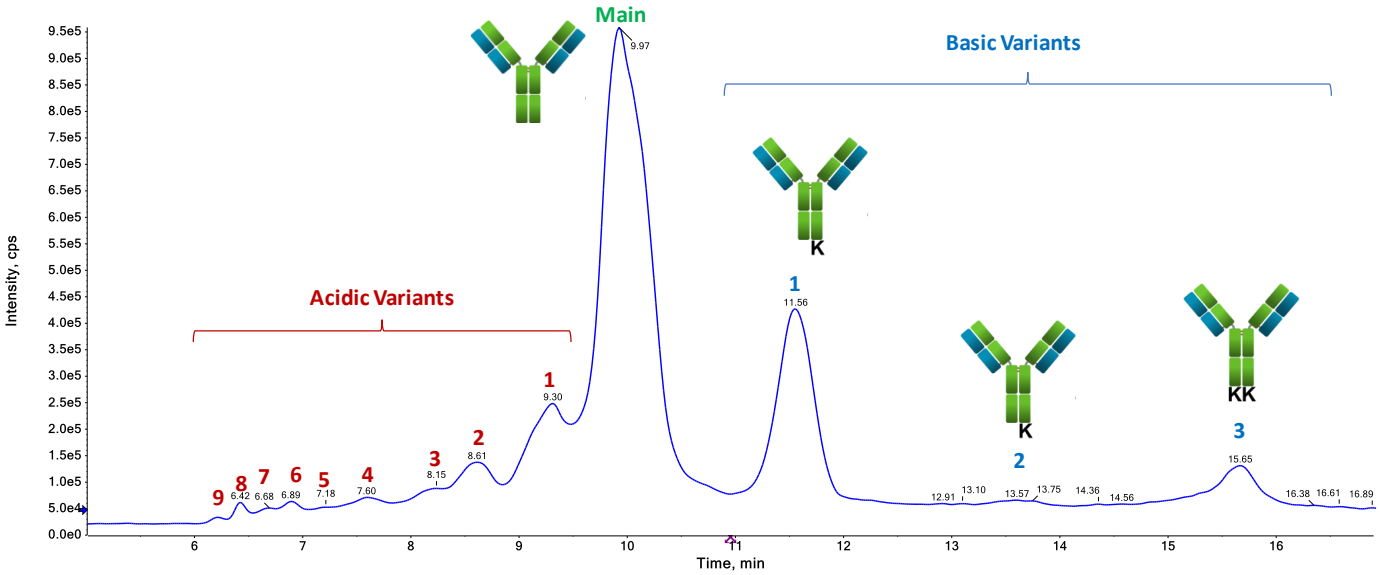

Figure 1. Total ion chromatogram of charge heterogeneity separation of adalimumab. The separation of a adalimumab antibody control sample using CEX resulted in the detection of the main species, 9 acidic variants and 3 basic variants. The main peak consists of the antibody without C-terminal lysine. Basic peak 1 and 2 is from adalimumab with one C-terminal lysine and basic peak 3 is from adalimumab with two C-terminal lysine residues.

Methods

Sample preparation: Adalimumab monoclonal antibody (mAb) samples were incubated in Tris buffer (pH = 8.4) at 42 °C for 10 days in total. 200 µg of samples were taken out and stored at -20 °C after 0 days (control sample), 1 day, 2 days, 3 days, 7 days and 10 days of stress. The samples were diluted to a concentration of 2 µg/µL with water before analysis.

Cation exchange chromatography: Mobile phase A consisted of 20 mM ammonium acetate (NH_4Ac), pH 5.2. Mobile phase B consisted of 5 mM ammonium acetate (NH_4Ac), pH 10.2. Separation was accomplished using an ExionLC™ System fitted with a Phenomenex bioZen™ 6 µm WCX column (150×2.1 mm) at 30°C using the gradient shown in Table 1. 26 µg sample from each time point was injected onto the LC/MS system.

Table 1. LC conditions for intact CEX analysis.

Time (min)	%A	%B	Flow Rate (mL/min)
Initial	*80*	*20*	*0.3*
1.00	*80*	*20*	*0.3*
16.00	*50*	*50*	*0.3*
16.01	*0*	*100*	*0.3*
18.55	*0*	*100*	*0.3*
18.56	*20*	*80*	*0.3*
29.00	*20*	*80*	*0.3*

Mass spectrometry: A SCIEX X500B QTOF System with a Turbo V™ Ion Source fitted with a Twin Sprayer ESI Probe was used for data acquisition. MS instrument conditions are listed in Table 2.

Table 2. MS parameters.

Parameter	Setting
Scan Mode	*Positive*
Gas 1	*60 psi*
Gas 2	*60 psi*
Curtain Gas	*35 psi*
Interface Heater Temperature	*650 °C*
Time Bins to Sum	*100*
Ionspray Voltage	*5500 V*
Accumulation Time	*0.3 sec*
CAD gas	*7*
Mass Range	*2,500 - 10,000 m/z*
Declustering Potential	*280 V*
Collision Energy	*10*

Data processing: Data were processed using the Analytics module within SCIEX OS Software 1.7 with intact reconstruction feature.

Charge heterogeneity assessment

High pH and elevated temperature stress is a common liability procedure employed in the biopharmaceutical industry, known to induce deamidation in antibodies.[4] In this liability study, adalimumab was used to showcase the application of an online CEX separation coupled to the SCIEX high resolution X500B QTOF System. The elution off a CEX column can either be achieved by increasing the ionic strength (increasing the salt concentration) or altering the pH. In this case, the 20 min LC separation was developed based on an ammonium acetate pH gradient to ensure compatibility with the subsequent MS analysis. The published range of isoelectric points (pI) of different adalimumab variants from 7.94-9.14 was considered for the method development.[5] The sample set consisted of samples stressed for different lengths of time as well as a control sample, which did not undergo any stress (Figure 1). All samples were analyzed using the CEX-MS method established, starting at a pH well below the lowest reported pI in order to enable the binding of the positively charged analytes to the negatively charged surface of the CEX column. With increasing pH, charge variants will be eluting regarding their pI, from lower towards higher pI.

A well resolved separation was achieved, based on the difference in charge of the analytes (Figure 1). The chromatogram showed nine acidic, three basic and the main variant, which is consistent with previously published data.[6] An excellent spectra quality was achieved under native-like conditions allowing for peak identification (Figure 2). During native-like analysis, proteins take up fewer charges compared to reversed phase conditions, for example. This results in less distribution of the signal and an increased spacing of different features of the sample on the *m/z*-axis. In addition, native-like analysis is less prone to induce artifacts during the analysis, which is especially desired when analyzing the charge heterogeneity of a biotherapeutic.

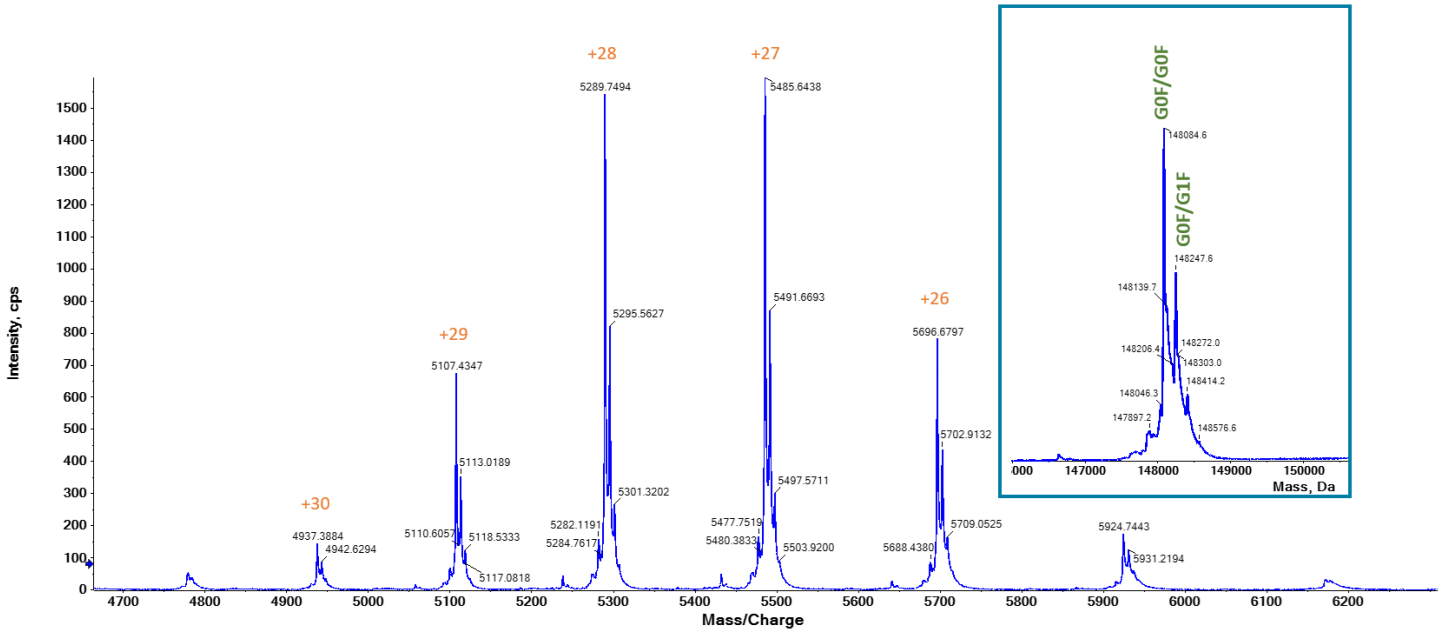

Figure 2. Data on main peak of control sample of adalimumab. Raw mass spectrum and reconstructed spectrum (insert) of major form showing G0F/G0F and G0F/G1F profiling.

Row	IS	Group	Name	Expected MW (Da)	m/z Range for XIC (Da)	Retention Time (min)	Reconstruction Start Mass (Da)	Reconstruction Stop Mass (Da)	IS Name	Experiment Index
1	☐	Adalimumab	Main	148084.00	2500 - 10000	9.97	145000.00	160000.00		1 +TOF MS (2500 - 10000)
2	☐	Adalimumab	Acidic 1	148087.00	2500 - 10000	9.30	145000.00	160000.00		1 +TOF MS (2500 - 10000)
3	☐	Adalimumab	Acidic 2	148089.00	2500 - 10000	8.61	145000.00	160000.00		1 +TOF MS (2500 - 10000)
4	☐	Adalimumab	Acidic 3	148092.00	2500 - 10000	8.00	145000.00	160000.00		1 +TOF MS (2500 - 10000)
5	☐	Adalimumab	Acidic 4	148096.00	2500 - 10000	7.47	145000.00	160000.00		1 +TOF MS (2500 - 10000)
6	☐	Adalimumab	Basic 1	148215.00	2500 - 10000	11.56	145000.00	160000.00		1 +TOF MS (2500 - 10000)
7	☐	Adalimumab	Basic 3	148342.00	2500 - 10000	15.65	145000.00	160000.00		1 +TOF MS (2500 - 10000)
8	☐									

Figure 3. Attribute definition for tracking of different charge variants in SCIEX OS Software 1.7. For tracking purposes, the main glycoform G0F/G0F was used for all charge variants.

Based on the reconstructed mass and the theoretical mass information, it was concluded that the main adalimumab peak corresponded to the antibody with no C-terminal lysine attached (Figure 2). The two most abundant glycoforms were identified as G0F/G0F and G0F/G1F (Figure 2). The main species was following a group of acidic variants (Figure 1). Besides sialylation (+291 Da), deamidation (+0.98 Da) is another major form contributing to acidic variants.[7] These two modifications cannot be distinguished based on the CEX profile alone, however, with the underlying MS information, sialyation could be ruled out as a possible cause, since the acidic variant peaks only showed a minor mass difference of a few Da compared to the main peak. The small mass shift caused by deamidation events is difficult to be differentiated from the unmodified species using intact mass analysis on a molecule of around 150 kDa. However, deamidation can lead to an increase in the net negative charge on the molecule and therefore to a decrease in pI values, leading to an earlier elution time compared to the main species.[7] The observed small mass shift on each acidic variant compared to the main species, aligns well with this previously published conclusion of deamidation events.[7,8] In addition, all acidic peaks showed a substantial increase with prolonged exposure of pH and temperature stress (Figure 8), which is in alignment with the potential cause being deamidation. Further identification of the two most abundant basic variants (basic peak 1 and 3) was achieved: basic 1 corresponded to the antibody with one C-terminal lysine and basic 3 corresponded to the antibody with

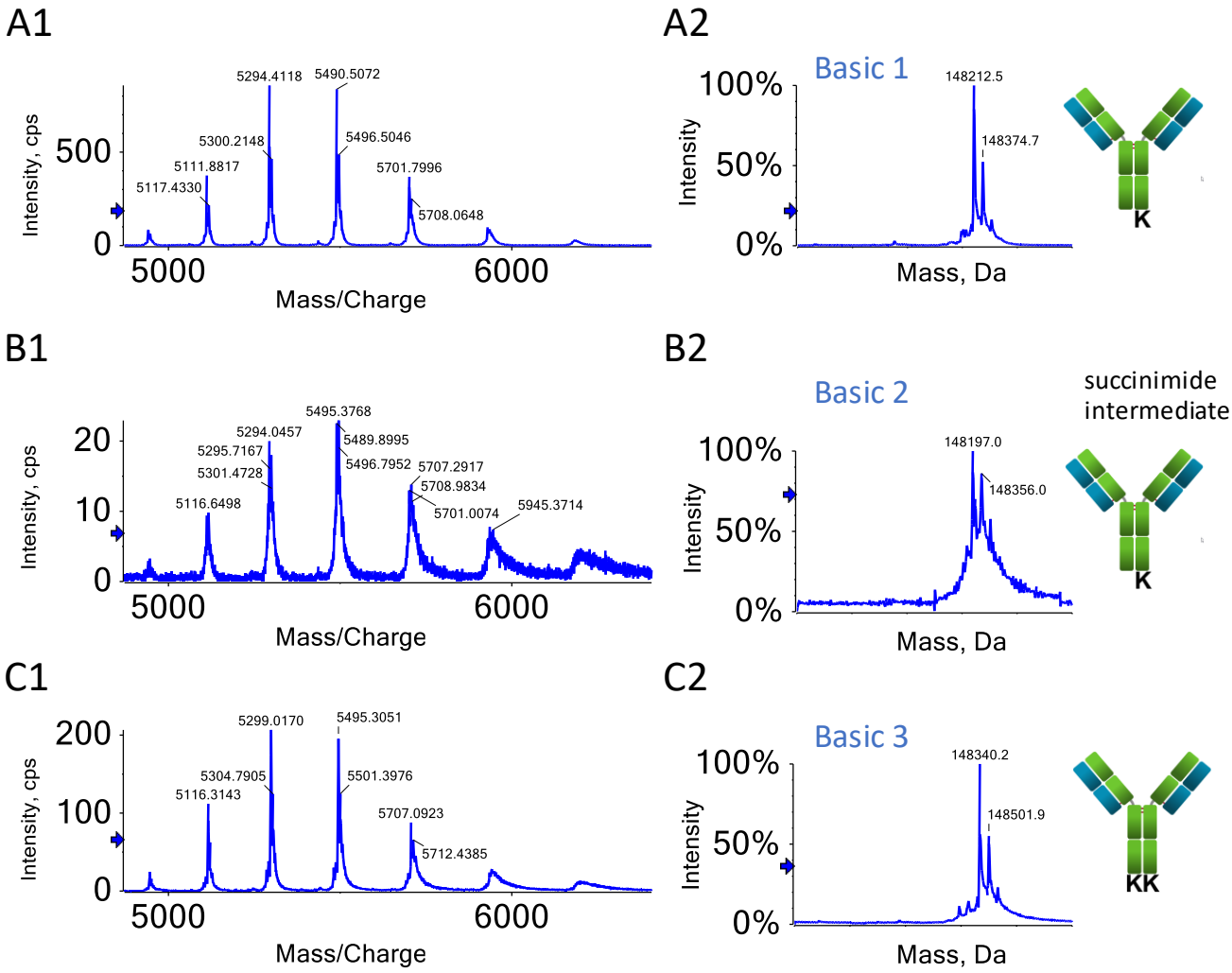

Figure 4. Data for basic variants of control sample of adalimumab. TOF-MS raw data and reconstructed data of basic 1 (A1, A2) being linked to the antibody with one C-terminal K, basic 2 (B1, B2) being linked to the mAb with one C-terminal K and a succinimide intermediate whereas basic 3 (C1, C2) was linked to the mAb with two C-terminal K.

Table 3. Summary of basic variants in adalimumab control sample.

variant	Theoretical mass (Da)	Experimental mass (Da)	Δ mass (Da)
Basic 1	*148208.2*	*148212.5*	*4.3*
Basic 2	*148192.2*	*148197.0*	*4.8*
Basic 3	*148336.4*	*148340.2*	*3.8*

two C-terminal lysines (Figure 4 and Table 3). Each lysine introduces one additional positive charge to the molecule, resulting in the formation of basic species with a higher pI. With the method setup being used (CEX), species with more positive charges (higher pI) are eluting later (at a higher pH) compared to those with less positive charges (lower pI). Basic variant 2 showed a mass of 148197 Da, which can correspond to a water loss (Δ mass = 16 Da) of basic variant 1. It is likely that this loss was caused by a succinimide intermediate being present in the basic variant 2 instead of an aspartic acid, causing a higher pI and therefore later elution time, which is in alignment with previous published data.[6]

The four most abundant acidic variants, the main variant and the two most abundant basic variants were employed to demonstrate the application of the intact MAM solution for CEX separation. With G0F/G0F being the dominant glycoform in

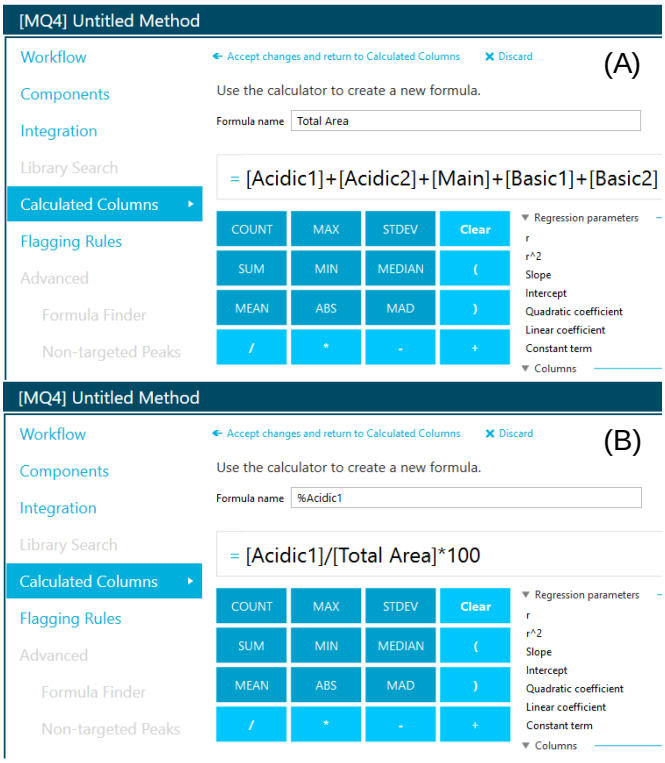

Figure 5. Example for definition of calculations. (A) Sum of all peak areas from different charge variants (B) Percentage calculation for each variant.

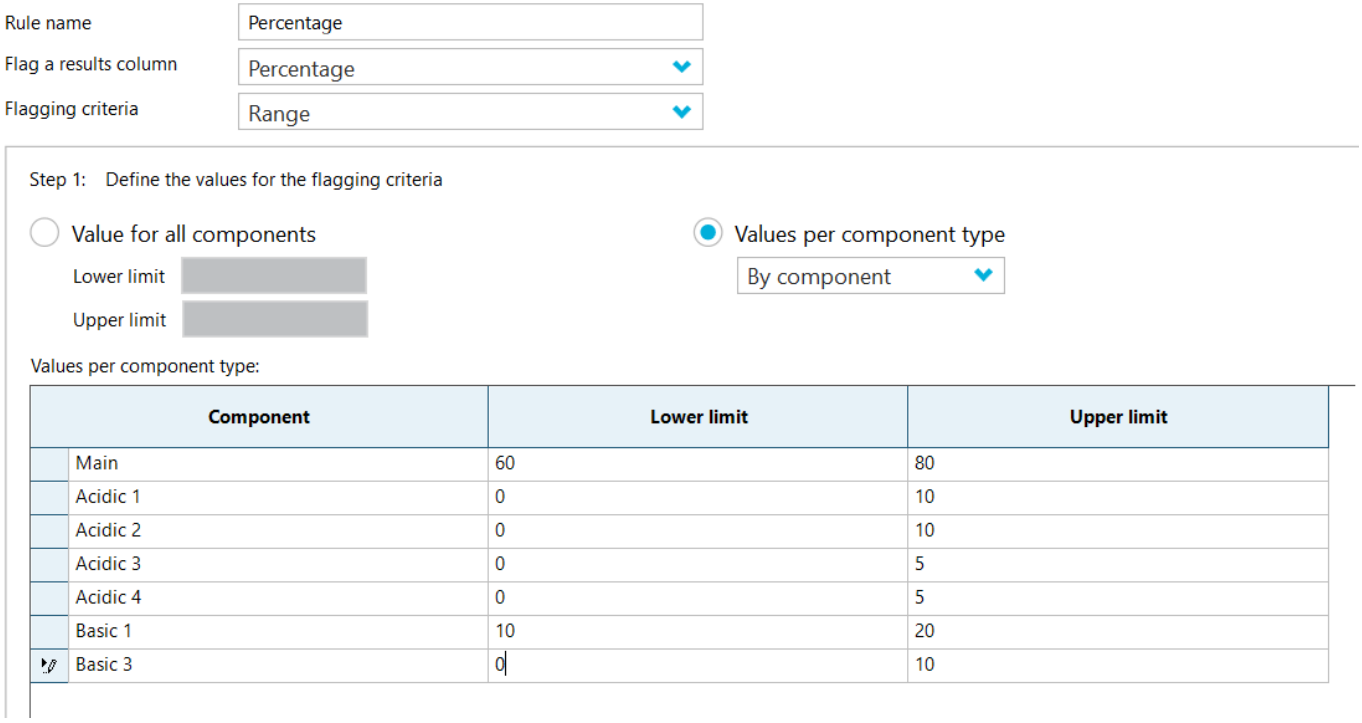

Figure 6. Definition of assay acceptance criteria. Users can choose from a range of flagging options. In this case a percentage range was used for flagging. For each component different flagging rules were defined.

adalimumab, the abundance of this proteoform was used for each charge variant for monitoring purposes (Figure 2 and 3).

Within the software, a reconstruction was performed, followed by a user-defined calculation (Figure 5). The reconstructed peak area of each charge variant is summed (Figure 5A) and the percentage for each charge variant is calculated respectively (Figure 5B) for each attribute defined. In this assay, the acceptance criteria were specified for each charge variant independently (Figure 6). For example, the acceptance criteria of the main peak was set to be between 60% to 80% of the total reconstructed peak area, while those of acidic 1 and 2 were set to below 10%, acidic 3 and 4 were set to below 5%, the basic 1 was set to be between 10% to 20% and the basic 3 was set to below 10%. Automatic color coding within the software allows a user to quickly understand which attributes were out of the user-specified range (Figure 7): red highlighting represents values above, while blue highlighting demonstrates values below the set criteria.

All samples derived from different time points of incubation were submitted for batch processing using the MAM assay developed, for automatic and quick processing of the MS data. Detailed result tables can be obtained and customized for accelerating data review (Figure 7). The results can be sorted by sample, targeted attribute or by the modification event. A metric plot can be generated to demonstrate percentage change of each attribute, which offers a great visualization tool in order to quickly understand changes in the molecule (Figure 8). The underlying data (chromatogram, raw MS data and reconstructed data) of each variant can be reviewed by clicking into each of the component lines, as shown previously.[9,10] Providing access to all relevant information in the same interface ensures confidence in correct and accurate identification and integration during streamlined data review.

Conclusions

- The coupled CEX-MS enables a great separation of adalimumab charge variants and excellent MS data quality under native conditions for assignment of different species
- The SCIEX flexible solution for MAM within SCIEX OS Software 1.7 provides a breakthrough in intact MAM analysis by offering a streamlined and compliant software package, from data acquisition through data analysis
- The combination of advanced hardware and streamlined software presents a cutting-edge solution for attribute monitoring in process development enabling faster decision making

[MQ4] Results Table (20200708 CEX Adalimumab.qsession)

42 rows | Filters: 0 | Qua | More

Index	Sample Name	Component Name	Component Group Na...	Expe... RT	Area	Rete... Time	Expected MW	MW	*Sumarea	% calc.
1	Control	Main	Adalimumab	9.97	8.900e4	9.91	148084.00	148084.63	120713.819	73.729
2		Acidic 1	Adalimumab	9.30	4.606e3	9.31	148087.00	148085.64	120713.819	3.816
3		Acidic 2	Adalimumab	8.61	1.650e3	8.63	148089.00	148093.01	120713.819	1.367
4		Acidic 3	Adalimumab	8.00	4.656e2	8.18	148092.00	148092.92	120713.819	0.386
5		Acidic 4	Adalimumab	7.47	N/A	7.61	148096.00	N/A	120713.819	0.000
6		Basic 1	Adalimumab	11.56	2.186e4	11.56	148215.00	148213.07	120713.819	18.109
7		Basic 3	Adalimumab	15.65	3.131e3	15.67	148342.00	148340.83	120713.819	2.594
8	Day 1	Main	Adalimumab	9.97	8.471e4	9.91	148084.00	148084.78	125330.932	67.587
9		Acidic 1	Adalimumab	9.30	1.321e4	9.26	148087.00	148086.61	125330.932	10.542
10		Acidic 2	Adalimumab	8.61	2.034e3	8.60	148089.00	148089.00	125330.932	1.623
11		Acidic 3	Adalimumab	8.00	9.294e2	8.15	148092.00	148092.87	125330.932	0.742
12		Acidic 4	Adalimumab	7.47	N/A	7.57	148096.00	N/A	125330.932	0.000
13		Basic 1	Adalimumab	11.56	2.161e4	11.54	148215.00	148213.87	125330.932	17.244
14		Basic 3	Adalimumab	15.65	2.836e3	15.61	148342.00	148340.78	125330.932	2.263
15	Day 2	Main	Adalimumab	9.97	7.828e4	9.91	148084.00	148084.77	123626.550	63.317
16		Acidic 1	Adalimumab	9.30	1.690e4	9.22	148087.00	148086.01	123626.550	13.674
17		Acidic 2	Adalimumab	8.61	3.045e3	8.58	148089.00	148088.08	123626.550	2.463
18		Acidic 3	Adalimumab	8.00	1.535e3	8.09	148092.00	148091.67	123626.550	1.242
19		Acidic 4	Adalimumab	7.47	7.357e2	7.57	148096.00	148096.45	123626.550	0.595
20		Basic 1	Adalimumab	11.56	2.055e4	11.52	148215.00	148213.90	123626.550	16.621
21		Basic 3	Adalimumab	15.65	2.581e3	15.60	148342.00	148340.86	123626.550	2.088
22	Day 3	Main	Adalimumab	9.97	7.261e4	9.92	148084.00	148084.87	120803.536	60.106
23		Acidic 1	Adalimumab	9.30	2.022e4	9.22	148087.00	148086.13	120803.536	16.742
24		Acidic 2	Adalimumab	8.61	3.899e3	8.56	148089.00	148088.42	120803.536	3.227
25		Acidic 3	Adalimumab	8.00	1.972e3	8.08	148092.00	148089.51	120803.536	1.633
26		Acidic 4	Adalimumab	7.47	1.025e3	7.55	148096.00	148097.63	120803.536	0.849
27		Basic 1	Adalimumab	11.56	1.891e4	11.52	148215.00	148214.36	120803.536	15.652
28		Basic 3	Adalimumab	15.65	2.163e3	15.60	148342.00	148340.56	120803.536	1.791
29	Day 7	Main	Adalimumab	9.97	4.778e4	9.98	148084.00	148085.58	110967.235	43.062
30		Acidic 1	Adalimumab	9.30	2.710e4	9.19	148087.00	148086.69	110967.235	24.419
31		Acidic 2	Adalimumab	8.61	1.146e4	8.54	148089.00	148088.51	110967.235	10.324
32		Acidic 3	Adalimumab	8.00	3.579e3	8.00	148092.00	148089.25	110967.235	3.225
33		Acidic 4	Adalimumab	7.47	1.994e3	7.52	148096.00	148093.49	110967.235	1.797
34		Basic 1	Adalimumab	11.56	1.732e4	11.56	148215.00	148217.97	110967.235	15.607
35		Basic 3	Adalimumab	15.65	1.737e3	15.56	148342.00	148341.66	110967.235	1.566
36	Day 10	Main	Adalimumab	9.97	3.415e4	10.03	148084.00	148085.89	102932.091	33.176
37		Acidic 1	Adalimumab	9.30	2.901e4	9.21	148087.00	148086.96	102932.091	28.188
38		Acidic 2	Adalimumab	8.61	1.801e4	8.58	148089.00	148088.84	102932.091	17.496
39		Acidic 3	Adalimumab	8.00	6.785e3	8.06	148092.00	148090.87	102932.091	6.592
40		Acidic 4	Adalimumab	7.47	2.775e3	7.54	148096.00	148091.72	102932.091	2.696
41		Basic 1	Adalimumab	11.56	1.096e4	11.56	148215.00	148219.21	102932.091	10.648
42		Basic 3	Adalimumab	15.65	1.241e3	15.54	148342.00	148342.52	102932.091	1.206

Figure 7. Attribute monitoring in SCIEX OS Software 1.7 utilizing reconstructed masses. Data summary in table format with each entry being linked to underlying chromatographic, raw and reconstructed data. Red and blue highlighted percentages were out of user-specified range.

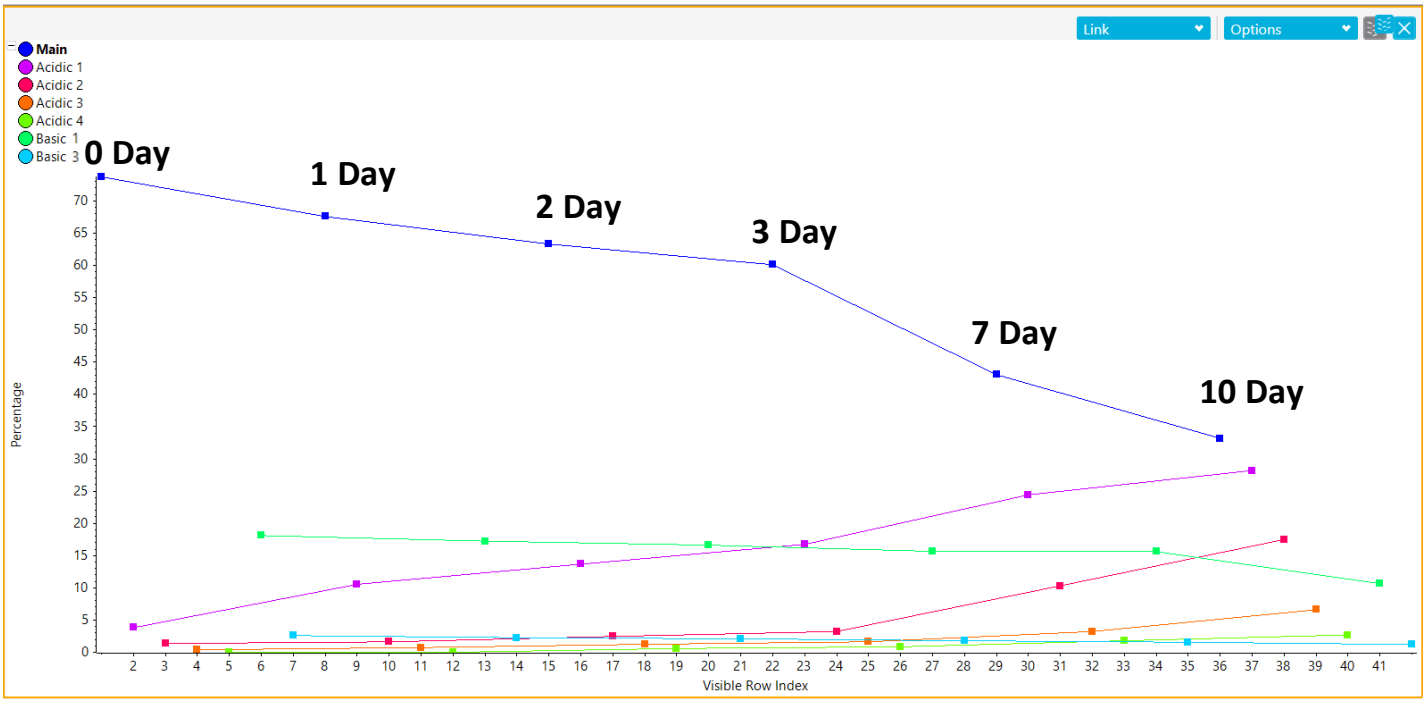

Figure 8. Metric plot showing high pH time course study. Trending of calculated percentage of respective species based on reconstructed peak area over incubation time for expedited data review. Adalimumab antibody was exposed to 42°C and pH 8.4 for different time periods as indicated. Color code indicates different charge variants.

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