Charge variant liability analysis using the SCIEX flexible solution for MAM

Microfluidic capillary electrophoresis (CE) separation via the ZipChip System with the SCIEX TripleTOF® 6600+ LC-MS/MS System

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In recent years, multiple attribute methodologies (MAM) have gained popularity in monitoring the product quality attributes (PQAs) of biotherapeutics within late-stage development and manufacturing laboratories.[1] Current MAM solutions use reversed-phase liquid chromatographic (RPLC) separation, which can provide separation based on hydrophobic affinity, however, it lacks information about a product's charge heterogeneity.

The charge heterogeneity profile can be classified as a critical quality attribute (CQA) if it has an impact on a therapeutics' safety and/or efficacy. Therefore a full understanding and monitoring of the variety of modifications which can lead to charge heterogeneity such as sialylation, deamidation, oxidation, glycation, C-terminal lysine, etc is desired. Standalone CE assays have been traditionally adopted to obtain this information in development, production and lot release. Recently, coupling CE separation with high resolution mass spectrometry has attracted extensive attention in the biopharmaceutical industry as it provides both, separation and identification of charged variants, in a single injection. However, accurate quantitation of each charge variant remains a challenge. With the launch of the SCIEX flexible solution for MAM custom product quality attribute (PQA) definition, monitoring and quantification utilizing CE-MS data, becomes integrated and easy.

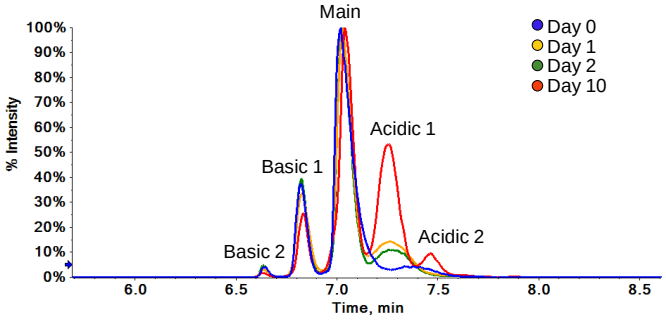
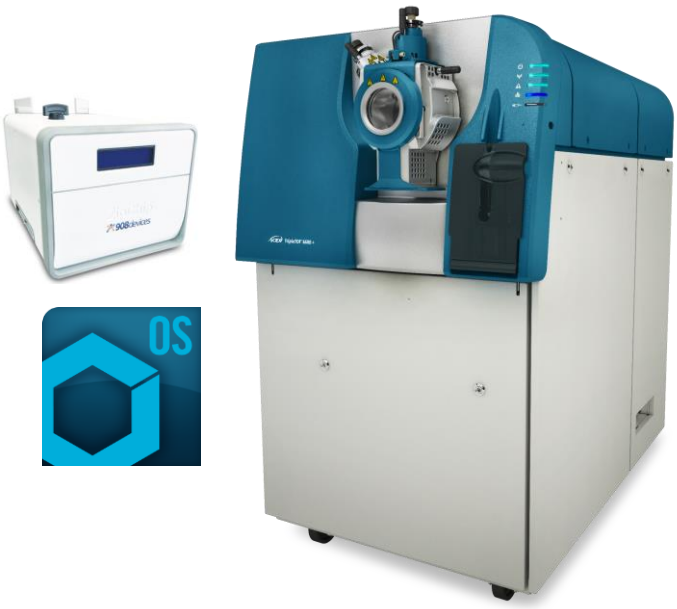


Figure 1. Overlaid electropherograms of charge heterogeneity separation. Adalimumab antibody exposed to 40°C and pH 8.4 for different time periods as indicated.



Here, a liability study focusing on charge heterogeneity separation (Figure 1) via the microfluidic ZipChip System in combination with the SCIEX TripleTOF® 6600 System is demonstrated. The SCIEX flexible solution for MAM within SCIEX OS Software 1.7 is employed to track the changes on intact reconstructed data for each charge variant during the liability analysis.

Key Features of SCIEX MAM solution

- Complete quantitative solution to track attribute changes observed using CE separation in combination with high resolution mass spectrometry
- Fast, reproducible and high-quality data acquisition with limited optimization of the CE and MS methods
- Great separation of different charged isoforms with MS compatible, ready-to-use buffer systems allowing for an additional dimension of information via MS

Methods

Sample Preparation: Adalimumab monoclonal antibody (mAb) samples were incubated in Tris buffer (pH = 8.4) at 40°C for 10 days. 50 µg of samples were taken out at 1 day, 2 days and 10 days. Samples were diluted to a concentration of 0.5 µg/µL with water before analysis.

Capillary Electrophoresis: Separation was performed with the ZipChip System (908 Devices Inc.) with an optional autosampler. Here, a charge variant TOF Kit containing premixed buffers was used. The chip consists of a separation channel and a built-in electrospray emitter (Figure 2). The ZipChip interface is available in a format compatible with SCIEX TripleTOF® 6600+ Systems. For each sample 1 nL (0.5 ng) were loaded onto the chip and separated at 500 V/cm. The total analysis time was set to 10 min with pressure assistance turned on at 0.5 min.

Mass Spectrometry: A TripleTOF® 6600+ System with an OptiFlow™ interface was used for data acquisition. Data were acquired using TOF MS mode. Detailed MS instrument conditions are listed in Table 1.

Data Processing: Data were processed using the Analytics module within SCIEX OS Software 1.7.

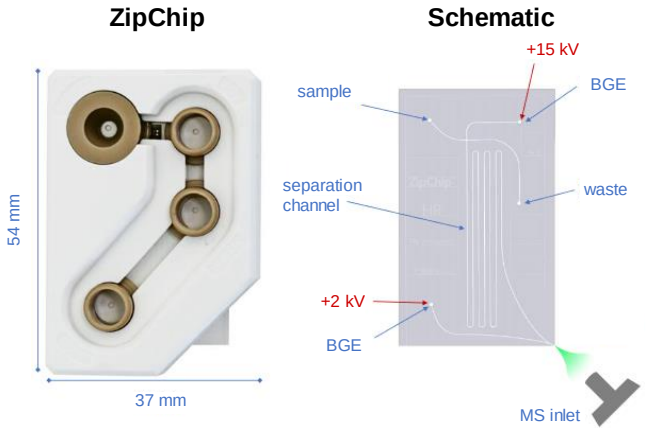


Figure 2. Chip of the ZipChip System. (Left) Actual chip with reservoirs for background electrolyte (BGE), sample and waste. (Right) Schematic of the chip showing an example of the separation channel.

Table 1. MS Parameters.

Parameter	Setting
Scan Mode	*Positive*
Gas 1	*4 psi*
Gas 2	*0 psi*
Curtain Gas	*10 psi*
Interface Heater Temperature	*50 °C*
Time Bins to Sum	*100*
Accumulation Time	*0.3 sec*
Mass Range	*3,000 - 7,000 m/z*
Declustering Potential	*160 V*
Collision Energy	*80*

Charge Heterogeneity Assessment

High pH and elevated temperature stress is a common liability procedure employed in the biopharmaceutical industry, inducing deamidation on antibodies.[2] Adalimumab was used in this liability study to showcase the usability of the ZipChip device in combination with the TripleTOF® 6600+ System for charge heterogeneity profiling of monoclonal antibody therapeutics.

Initially, the samples generated by exposing the mAb to stress for different lengths of time were analyzed using RP-LC, but no difference was observed with intact mass analysis (data not shown). Subsequently, the same samples were analyzed using the CE-MS acquisition method developed in a previous technical note.[3] A well resolved separation of five peaks was achieved, based on difference in the size and charge of the analytes (Figure 1).

With the embedded mass spectrometry information, the identity of each peak can be further determined (Figure 3). The first peak at 6.6 min was identified as the antibody with two C-terminal lysines (2K), whereas the second peak (6.8 min) was linked to Adalimumab with one C-terminal lysine residue (1K) and the main form did not contain any C-terminal lysine.

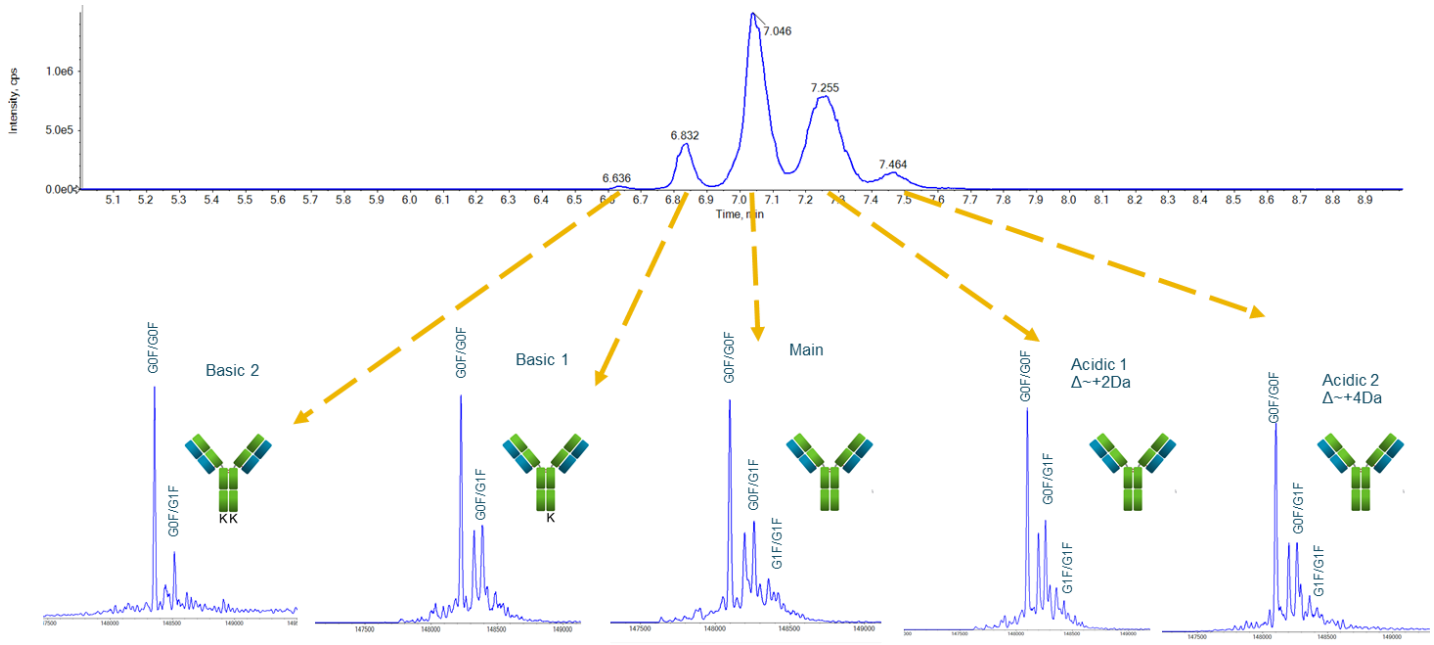


Figure 3. Data on pH and temperature stressed adalimumab mAb. (Top) Electropherogram of adalimumab sample stressed for 10 days at pH = 8.4 and 40°C. (Bottom) Reconstructed data for the five different peaks of the electropherogram, respectively. From left to right - basic variant 2 with two C-terminal K; basic variant 1 with one C-terminal K; main species without terminal K, acidic variant 1, acidic variant 2.

Each lysine introduces one additional positive charge on the molecule, resulting in an increase in the isoelectric point (pI). With the method setup being used, the migration time of species with a higher pI will be shorter than those with a lower pI. The main species without any C-terminal lysine residue (0 K) was followed by a group of acidic variants (Figure 3, top). Both acidic variant peaks show a small mass difference compared to the main peak, which can be caused by deamidation for instance. With prolonged exposure of pH and temperature stress, both acidic peaks show a substantial increase (Figure 1 and Figure 6 blue and pink trace), which is in alignment with the potential cause being deamidation.

With G0F/G0F being the dominant glycoform in adalimumab, the abundance of this proteoform was used for each charge variant for monitoring purposes (Figure 4). Within the software, a reconstruction is 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 independently specified. For example, the acceptance criteria of the main peak was set to above 60% while that of acidic peak 1 was set to below 10%. Automatic color coding within the software offers a user to quickly understand which attributes were out of the specified range (Figure 6).

All samples derived from different time points of incubation were submitted for batch processing using the MAM assay developed.

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 Na...	Experiment Index
1	☐	Basic 2	G0F/G0F+2Lys	148357.00	3000 - 5000	6.61	145000.00	155000.00		1 TOF MS (3000.0 - 7000...
2	☐	Basic 1	G0F/G0F+1Lys	148227.00	3000 - 5000	6.84	145000.00	155000.00		1 TOF MS (3000.0 - 7000...
3	☐	Main	G0F/G0F	148098.40	3000 - 5000	7.03	145000.00	155000.00		1 TOF MS (3000.0 - 7000...
4	☐	Acidic 1	G0F/G0F acidic 1	148099.90	3000 - 5000	7.43	145000.00	155000.00		1 TOF MS (3000.0 - 7000...
5	☐	Acidic 2	G0F/G0F acidic 2	148102.30	3000 - 5000	7.48	145000.00	155000.00		1 TOF MS (3000.0 - 7000...

Figure 4. Attribute definition for main glycoforms G0F/G0F from different charge variants in SCIEX OS Software 1.7.

After data processing, SCIEX OS Software 1.7 provides a detailed results table for data review (example in Figure 6). 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 understand changes in the molecule quickly. The underlying data of each variant can be reviewed by clicking into each of the component lines. The electropherogram, raw spectrum and reconstructed data are available in the same interface to provide confidence in correct and accurate identification and integration during data review.

Deamidation events cause a small molecular weight increase of +0.98 Da per site, which is difficult to differentiate 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, which causes the formation of acidic variants.[4] Besides sialylation (+291 Da), deamidation is another major form contributing to acidic variants.[5] The observed small mass shift on each acidic variant compared to the main species, aligns well with this previously published conclusion of deamidation events.[2,4-5]

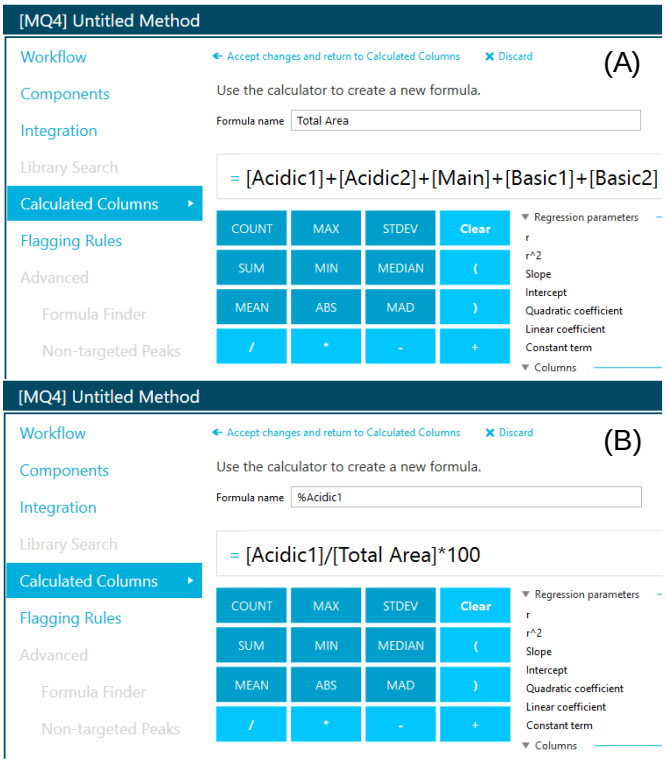


Figure 5. **Definition of calculations**. (A) Sum up all the peak area from different charge variants (B) Percentage calculation.

Conclusions

- The SCIEX flexible solution for MAM provides a breakthrough in intact MAM analysis by offering a streamlined and compliant software package, from data acquisition throughout data analysis
- The ZipChip system coupled to the TripleTOF® 6600 System enables a great separation of adalimumab charge variants and excellent MS data quality
- The combination of advanced hardware and streamlined software presents a cutting-edge solution for attribute monitoring in process development enabling faster decision making

Index	Sample Name	Component Na...	Component Group Name	Expec... RT	Area	Ret... Time	Expected MW	MW	*Total area	Acidic 1	Acidic 1	Main	Basic2	Basic1
2	Control	G0F/G0F+1Lys	Basic 2	6.84	4.909e4	6.82	148227.00	148226.97	217018.170	2.913	0.000	70.548	3.918	22.621
3		G0F/G0F	Neutral	7.03	1.531e5	7.02	148098.40	148098.60	217018.170	2.913	0.000	70.548	3.918	22.621
4		G0F/G0F acidic 1	Acidic 1	7.43	6.321e3	7.33	148099.90	148102.48	217018.170	2.913	0.000	70.548	3.918	22.621
5		G0F/G0F acidic 2	Acidic 2	7.71	N/A	7.61	148102.30	N/A	217018.170	2.913	0.000	70.548	3.918	22.621
6	1 day	G0F/G0F+2Lys	Basic 1	6.65	9.183e3	6.64	148357.00	148356.82	232863.107	8.733	0.129	63.295	3.944	23.899
7		G0F/G0F+1Lys	Basic 2	6.84	5.565e4	6.82	148227.00	148226.76	232863.107	8.733	0.129	63.295	3.944	23.899
8		G0F/G0F	Neutral	7.05	1.474e5	7.03	148098.40	148099.12	232863.107	8.733	0.129	63.295	3.944	23.899
9		G0F/G0F acidic 1	Acidic 1	7.30	2.034e4	7.26	148099.90	148101.99	232863.107	8.733	0.129	63.295	3.944	23.899
10		G0F/G0F acidic 2	Acidic 2	7.59	2.995e2	7.45	148102.30	148101.71	232863.107	8.733	0.129	63.295	3.944	23.899
11	2 days	G0F/G0F+2Lys	Basic 1	6.63	6.982e3	6.64	148357.00	148357.13	235600.094	12.330	0.335	64.143	2.963	20.227
12		G0F/G0F+1Lys	Basic 2	6.83	4.766e4	6.82	148227.00	148227.69	235600.094	12.330	0.335	64.143	2.963	20.227
13		G0F/G0F	Neutral	7.05	1.511e5	7.02	148098.40	148098.81	235600.094	12.330	0.335	64.143	2.963	20.227
14		G0F/G0F acidic 1	Acidic 1	7.31	2.905e4	7.26	148099.90	148101.00	235600.094	12.330	0.335	64.143	2.963	20.227
15		G0F/G0F acidic 2	Acidic 2	7.58	7.903e2	7.44	148102.30	148099.97	235600.094	12.330	0.335	64.143	2.963	20.227
16	10 days	G0F/G0F+2Lys	Basic 1	6.61	1.343e3	6.63	148357.00	148357.20	166089.763	37.871	6.132	43.034	0.809	12.155
17		G0F/G0F+1Lys	Basic 2	6.84	2.019e4	6.84	148227.00	148227.55	166089.763	37.871	6.132	43.034	0.809	12.155
18		G0F/G0F	Neutral	7.03	7.147e4	7.04	148098.40	148098.41	166089.763	37.871	6.132	43.034	0.809	12.155
19		G0F/G0F acidic 1	Acidic 1	7.26	6.290e4	7.26	148099.90	148099.95	166089.763	37.871	6.132	43.034	0.809	12.155
20		G0F/G0F acidic 2	Acidic 2	7.48	1.018e4	7.47	148102.30	148102.16	166089.763	37.871	6.132	43.034	0.809	12.155

Figure 6. Attribute monitoring in SCIEX OS Software 1.7 utilizing reconstructed masses. (A) Data summary in table format with each entry being linked to (C) underlying electropherogram, raw and reconstructed data. (B) Trending of calculated percentage of respective species based on reconstructed peak area over incubation time for expedited data review.

References

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4. Lyubarskaya Y, et al. (2006) Analysis of recombinant monoclonal antibody isoforms by electrospray ionization mass spectrometry as a strategy for streamlining characterization of recombinant monoclonal antibody charge heterogeneity. *Anal Biochem*. **348(1),** 24-39.
5. Khawli LA, *et al.* (2010) Charge variants in IgG1. Isolation, characterization, in vitro binding properties and pharmacokinetics in rats. *mAbs.* **2(6)**, 613-624.

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