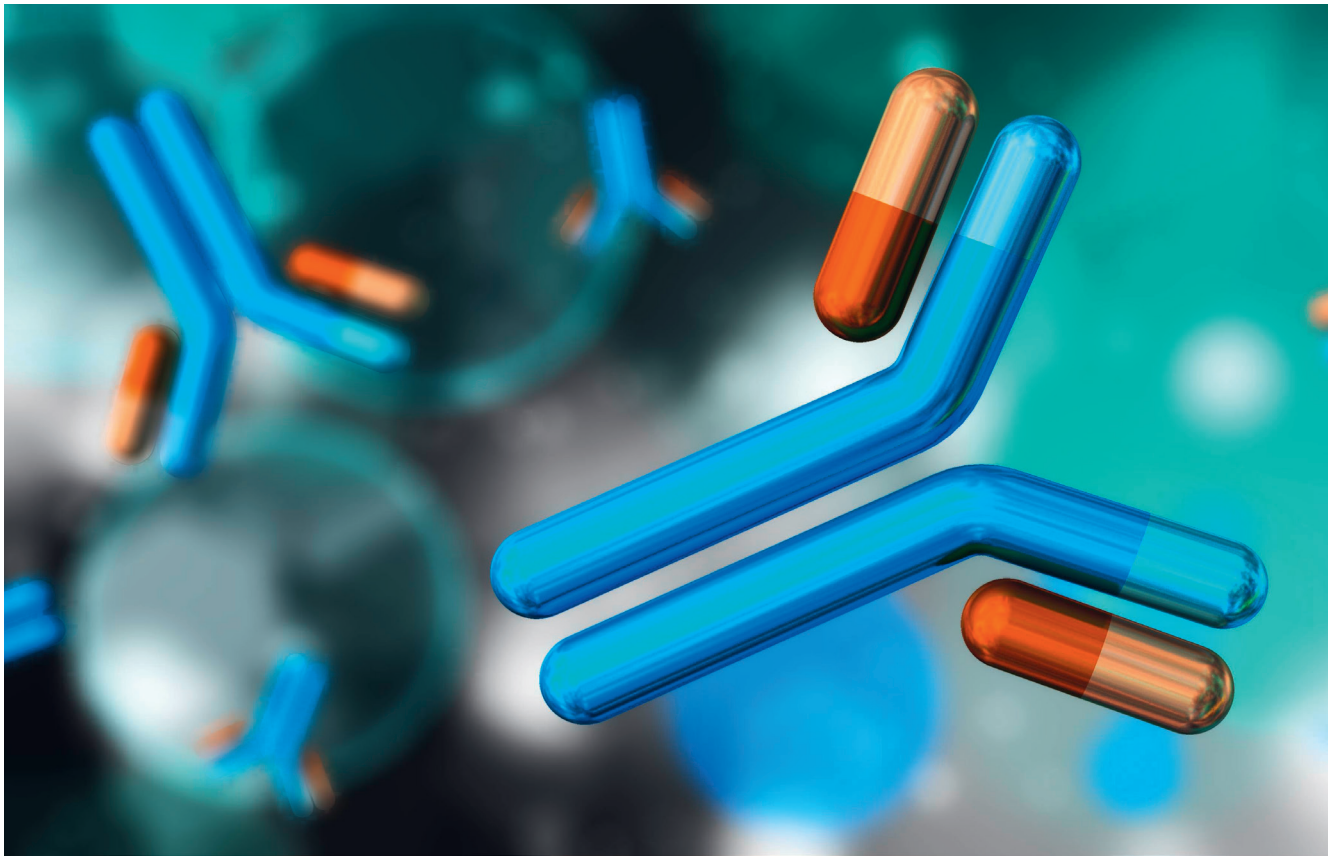
Utilisation of AEX-MS for charge variant analysis of IgG4-based mAbs!

Cation exchange chromatography (CEX) is an excellent method of characterising the charge heterogeneity of biomolecules. This is also true for most commercially available monoclonal antibodies (mAbs). They are often IgG1-based and possess a high isoelectric point (pI) of usually ≥8. Therefore, CEX coupled with mass spectrometry (MS) is the traditional approach [1]. In contrast, anion exchange chromatography (AEX) has only been used for relatively acidic proteins.
However, for IgG4-based mAbs AEX may be an alternative approach. They possess a pI<8 and therefore CEX is less suitable.



This technical note describes the successful application of an AEX method for charge heterogeneity analysis of IgG4-based mAbs coupled to a mass spectrometry (MS) detector. Several IgG4-based mAbs with different pIs (between 6.1–7.3) as well as NISTmAb (pI=9.2) were analysed using a strong anion exchange column (SAX), BioPro IEX QF [2].

Native AEX-MS method development

The combination of AEX and MS requires a special setup (see Figure 1) in which a stainless-steel T-piece after the column divides the flow. Most of the flow is directed to the UV detector, while the remaining sub-microlitre per minute flow is directed to the nanoelectrospray ionisation mass spectrometer (NSI-MS). NSI is used because it can tolerate high salt concentrations of up to 600 mM ammonium acetate. To further improve the spray stability, isopropanol is used as a dopant, modified desolvation gas.

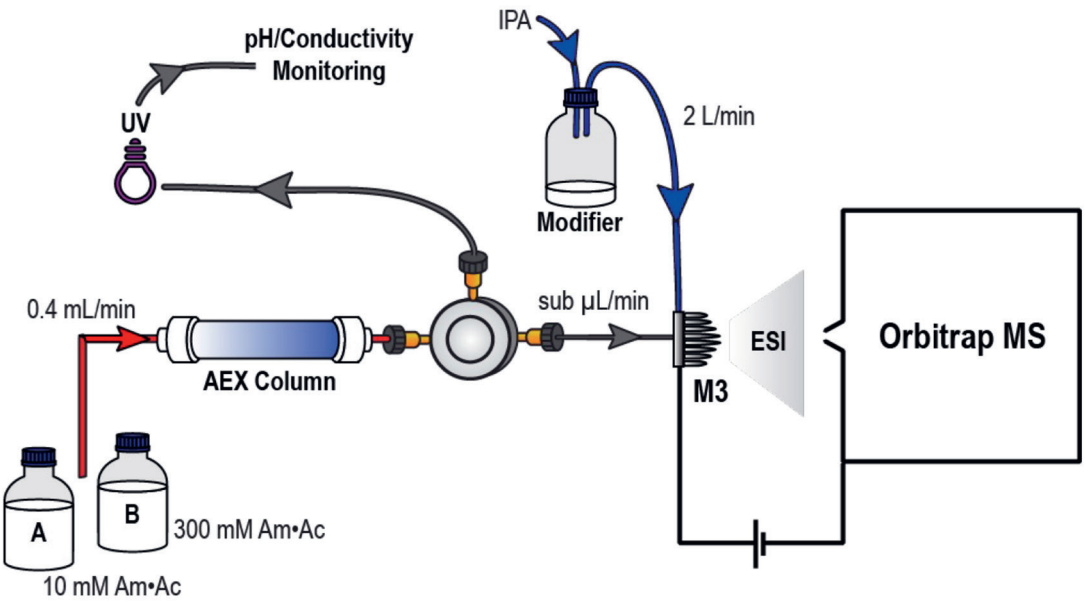


Figure 1: Native AEX-MS analysis before (blue) and after PNGase F-treatment (black) [2].

Table 1: Chromatographic conditions of the AEX-MS method [2].

Column:	BioPro IEX QF (5µm) 100 x 4.6 mm ID
Part No.:	QF00S05-1046WP
Eluent:	A) 10 mM ammonium acetate, pH 6.7
	B) 300 mM ammonium acetate, pH 6.8
Gradient:	0%B (0–2 min), 0–100%B (2–18 min), 100%B (18–22 min)
Flow rate:	0.4 mL/min
Temperature:	45 °C intact mAb
	25 °C subunit analysis
Injection:	5 or 10 µg mAb sample
Detection:	NSI-MS (nanoelectrospray ionisation)
	UV
Sample:	In-house IgG4-based mAbs (Regeneron)
	NISTmAb
Post column setup:	Post column stainless-steel T-piece to direct the majority to the UV detector
	Remaining sub-microlitre per minute flow directed to the NSI-MS

A salt gradient from 10 mM ammonium acetate (A) to 300 mM ammonium acetate (B) (pH unadjusted) is used to analyse an IgG4 based mAb (mAb-B) with a pI of 6.8. Different temperatures of 25, 35 and 45 °C are tested (see Figure 2). At 45 °C, variant separation is significantly improved and sharper peaks are obtained. Four basic and two acidic peaks of mAb-B can be detected.

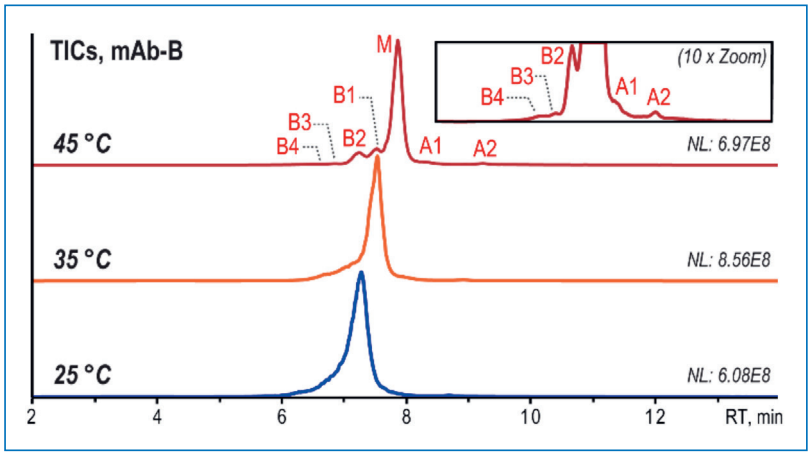


Figure 2: Native AEX-MS analysis of mAb-B (pI=6.8) at column temperatures of 25 °C (blue), 35 °C (orange), and 45 °C (red) [2].

These conditions were applied to various IgG4-based mAbs (pI 6.1–7.3) as well as to the NISTmAb, which is IgG1-based and has a pI of 9.2. Figure 3 shows that the AEX-MS method is suitable for IgG4-based mAbs with moderate pIs, but not for IgG1-based mAbs with higher pIs. The separation improves as the pI gets lower.

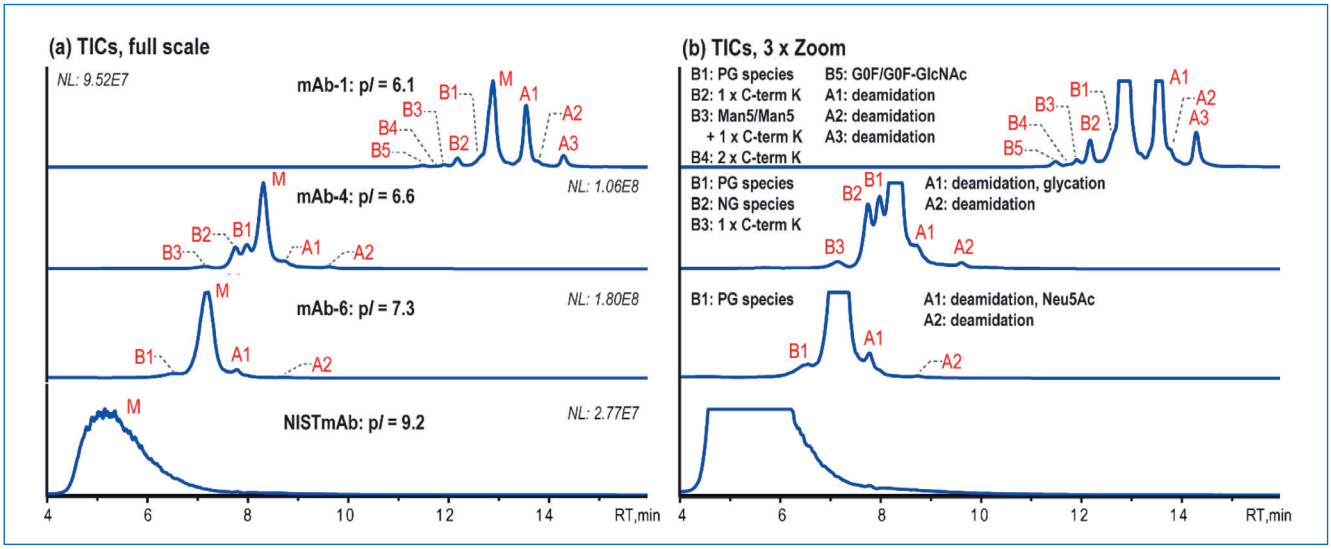


Figure 3: Native AEX-MS charge variant analysis of three different IgG4-based mAbs and NISTmAb, shown basic (B) and acidic (A) variant peaks as well as the main species (M), 5 µg injection [2].

mAb-6 has a pI of 7.3, which is higher than the mobile phase pH, but sufficient separation still occurs. This suggests that it is the surface charge rather than the intrinsic charge that causes the AEX-based separation. The acidic variants separated correlate with those commonly observed by CEX-MS such as deamidation, glycation and sialic acid (Neu5Ac)-containing species.

Deamidated variants were found in several peaks. The additional abundant peak A1 of mAb-1 contains deamidation that correlates with a known deamidation site in its complementary determining regions (CDR). However, the other mAbs tested have no deamidation sites in their CDRs, so these two acidic peaks are probably due to site-specific deamidation in the Fc region.

The basic variants observed can be identified as unprocessed C-terminal Lys (C-term K) and mAb species with different numbers of Fc N-glycans. In addition, the glycoforms Man5/Man5 with unprocessed C-terminal K and G0F/G0F-GlcNac are observed for mAb-1. mAb-4 demonstrates that this AEX-MS method is very sensitive to the macroheterogeneity of Fc N-glycosylation. The main fully glycosylated (FG) species is separated from the partially glycosylated (PG) peak B1 and the non-glycosylated (NG) species B2, which elute earlier.

Comparison of the AEX-MS method with CEX-MS

The IgG4-based mAb-B with a pI of 6.8 is used to compare the two methods (Figure 4). From the CEX-MS method, two acidic peaks can be observed and identified as deamidated and glycated variants. There are no basic peaks.

Table 2: Chromatographic conditions of the CEX-MS method [2].

Column:	BioPro IEX SF (5 µm) 100 x 4.6 mm ID
Part No.:	SF00S05-1046WP
Eluent:	A) 20 mM ammonium acetate, pH adjusted to 5.6 with 20 mM acetic acid
	B) 150 mM ammonium acetate, pH 6.8
Gradient:	0%B (0–2 min), 0–100%B (2–18 min), 100%B (18–22 min)
Flow rate:	0.4 mL/min
Temperature:	45 °C
Injection:	10 µg mAb sample
Detection:	NSI-MS (nanoelectrospray ionisation)
	UV
Sample:	In-house IgG4-based mAb, pI=6.8 (Regeneron)
Post column setup:	Post column stainless-steel T-piece to direct the majority to the UV detector
	Remaining sub-microlitre per minute flow directed to the NSI-MS

In comparison, the AEX-MS analysis shows four basic peaks including partially and non-glycosylated mAb species and 1 or 2 unprocessed C-terminal K in addition to two acidic peaks. However, A1 consists of a deamidated and glycated variant and A2 contains another deamidated species.

The use of AEX-MS probably provides the possibility to separate site-specific deamidation variants. The overall separation using AEX-MS is better as the method provides sharper peaks and additional information about the base variants.

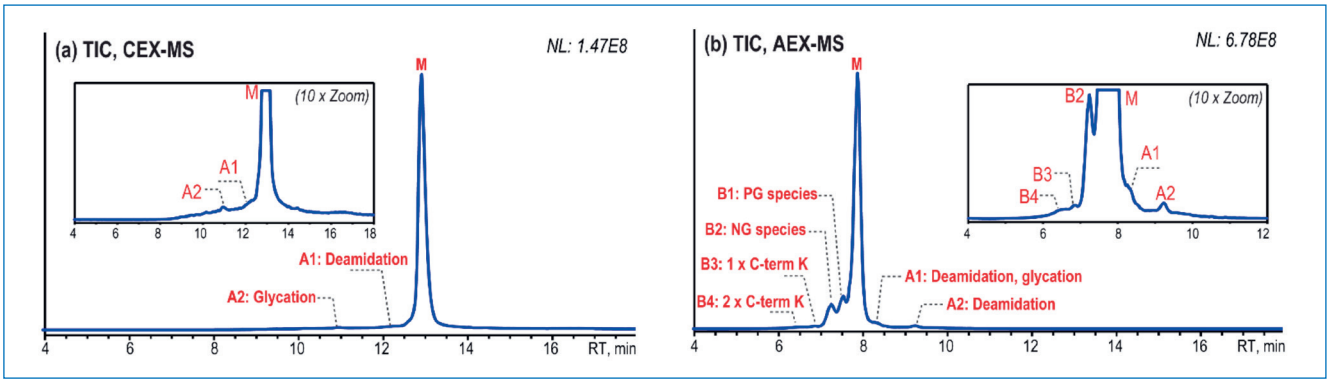


Figure 4: Comparison of (a) CEX-TIC and (b) AEX-TIC of an IgG4 mAb (pI=6.8) [2].

Further improvement of the basic variant separation

Since mAbs with a lower pI are better separated, it was tested to see whether the separation can be improved by lowering the pI through PNGase F (peptide:N-glycosidase F) -mediated deglycosylation. This reaction removes N-glycans and simultaneously converts the glycan-bearing asparagine (Asn) residue to aspartic acid (Asp). Since all IgG4 mAbs contain an Asn residue in the Fc region of each of the two heavy chains, up to two Asn to Asp conversions can be expected.

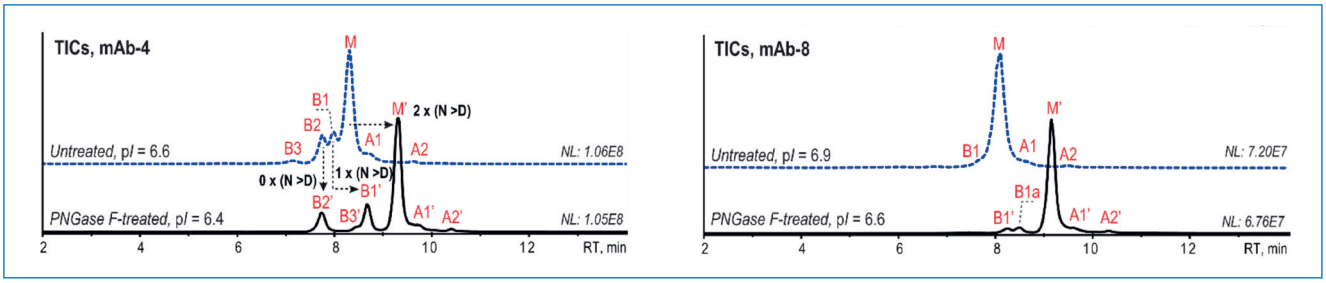


Figure 5: Native AEX-MS analysis of mAb-4 (right) and mAb-8 (left) before (blue) and after PNGase F-treatment (black) [2].

Figure 5 shows the differences between the untreated and the PNGase F-treated mAbs mAb-4 (pI=6.6) and mAb-8 (pI=6.9). The PNGase F-treatment decreased the pI to 6.4 for mAb-4 and 6.6 for mAb-8. Due to the reduced pI the overall retention as well as the variant separation and peak sharpness are improved for both mAbs.

After the PNGase F-treatment of mAb-4, the retention time of the FG main species increases by about 1 min due to the elevated acidity induced by the now two Asp residues. The retention time of the PG species B1 shifts by only about 0.5 min, while the retention time of the NG species B2 remains unchanged. In the case of mAb-8, an additional minor variant (B1a) can be detected and identified as a partially glycosylated species.

Monitoring critical Fc quality attributes

This AEX-MS method can also be used for subunit analysis of mAbs after digestion with IdeS protease. Since the pI of F(ab')2 fragments is relatively high, these fragments are poorly retained and are not taken into account further.
The Fc fragments were treated with PNGase F-mediated glycosylation to take advantage of the improved resolution and to further investigate the glycosylated species. In contrast to previous results, a lower temperature of 25 °C showed improved peak shape and charge variant separation for the Fc fragment analysis compared to analysis at higher temperatures, as shown in Figure 6 for mAb-4.

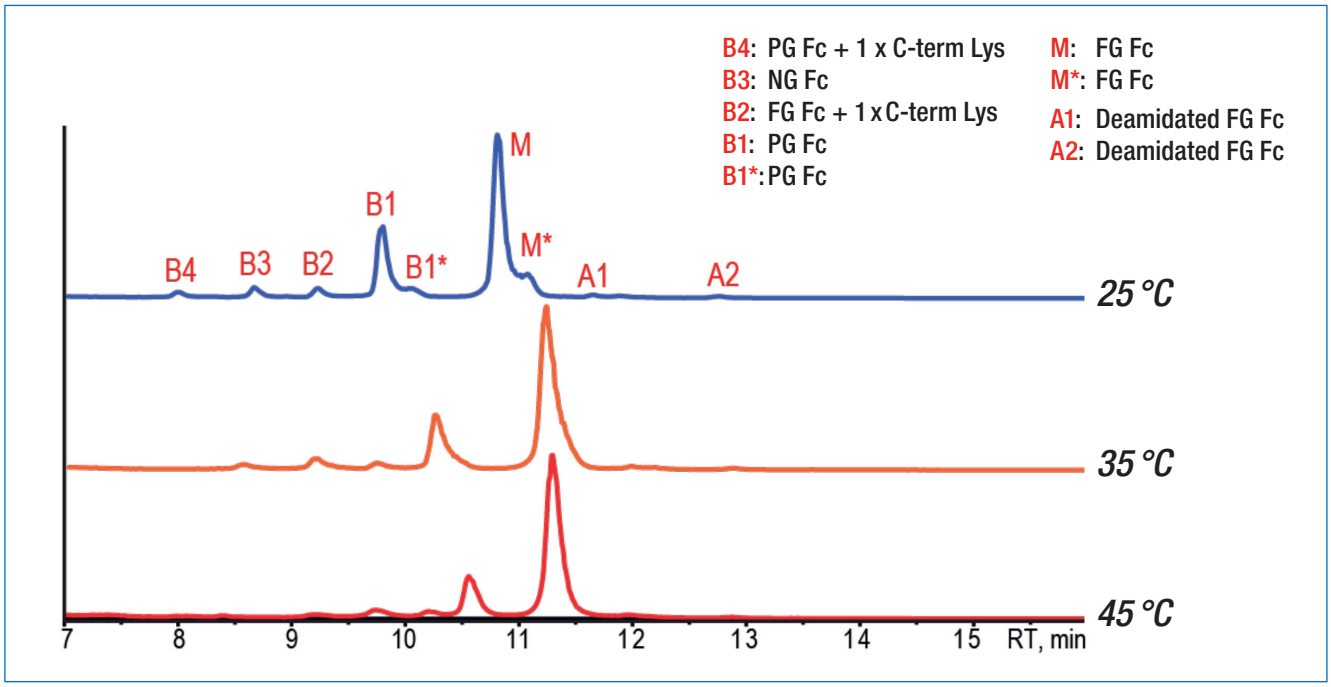


Figure 6: Native AEX-MS analysis of the PNGase F-treated and IdeS digested mAb at different temperatures: 25 °C (blue), 35 °C (orange) and 45 °C (red) [2].

Four basic and two acidic variants can be identified. The main peak as well as B1 show tailing shoulder peaks, with identical mass to the corresponding peak, which could be conformational isomers. In addition to the NG B3 and the PG B1 peak, B2 is identified as a fully glycosylated species with one unprocessed C-term Lys while B4 is identified as a partially glycosylated species with one unprocessed C-term Lys. These findings were confirmed by peptide mapping.

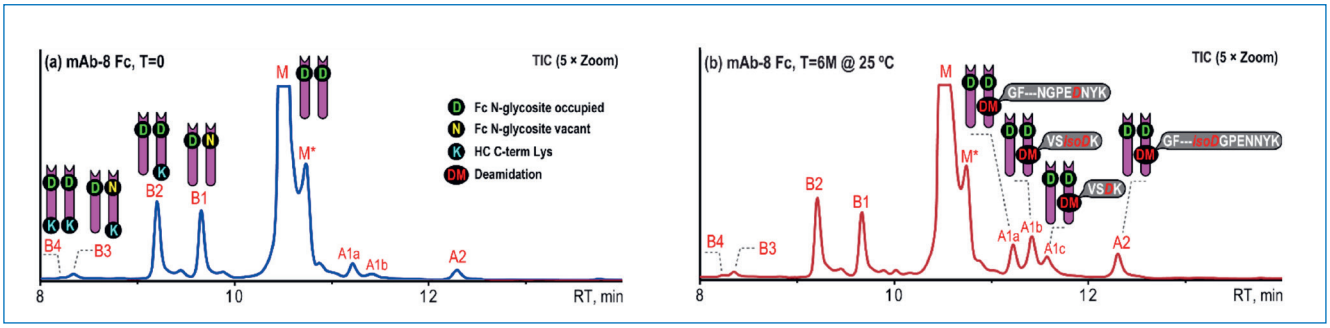


Figure 7: AEX-MS analysis of the PNGase F and IdeS-treated mAb-8 at (a) T=0, and (b) T = 6M @ 25 °C [2].

mAb-8 was thermally stressed to achieve higher levels of deamidation and to facilitate fractionation after IdeS digestion and deglycosylation. Therefore, mAb-8 was incubated at 25 °C for 6 months. The basic Fc variants of mAb-8 are also assigned to the unprocessed C-term K and Fc N-glycosylation macroheterogeneity. These basic variants did not change after thermal stress. While an increase in peaks for the acidic variants was observed, which can be completely attributed to deamidation.

Analysis of Fc fragments improved the resolution of separated deamidated variants. Four acidic peaks are resolved from the analysis of the thermally stressed mAb. Since there are only a few deamidation sites present in the IgG4 Fc region, it is likely that this AEX-MS method can separate site-specific deamidations.

Possible deamidation sites are NG at VVSVLTVLHQDWLNGK; NK at VSNK; NG and NN at GFYPSDIAVEWESNGQPENNYK. Therefore, the fractions of the deamidated variants were identified by peptide mapping. The results of the peptide mapping of the main and acidic fractions are shown in Table 3. It is noticeable that A1b and A1c both contain the deamidated VSNK peptide, but A1b mainly contains the isoAsp form, whereas A1c mainly contains the Asp form. It is striking that this AEX-MS method is capable of separating site-specific deamidation products at the Fc level, even at isoform resolution, so that these attributes can be monitored without the need for peptide mapping. This saves time and reduces sources of error such as deamidation artefacts that can occur in peptide mapping.

Table 3: Relative abundance of deamidated tryptic peptides from peptide mapping Analysis of the main and acidic (A1a, A1b, A1c, and A2) fractions from thermally stressed PNGase F treated and IdeS digested mAb-8 [2].

Deamidated Peptides	Relative Abundance (%)				
	Main Fraction	A1a Fraction	A1b Fraction	A1c Fraction	A2 Fraction
GFYPSDIAVEWESNGQPE***D***NYK	0.1	***7.3***	4.1	1.8	2.1
VS(***isoD***)K	0.3	0.0	***28.5***	15.1	0.0
VS***D***K	0.2	0.0	2.3	***17.7***	0.1
GFYPSDIAVEWES***D***GQPENNYK	0.2	0.1	0.1	0.1	2.0
GFYPSDIAVEWES(***isoD***)GQPENNYK	1.0	0.1	0.7	0.9	***23.9***

Summary

AEX-MS analysis is very suitable for characterising charge heterogeneity in IgG4-based mAbs. Compared to CEX, the AEX method shows overall better separation of the IgG4 based mAb with moderate pI and provides additional information.

The resolution of the glycosylated variants can be further improved by PNGase F-mediated deglycosylation.

AEX-MS methods are suitable for the Fc critical quality attribute monitoring of IgG4-based mAbs, while CEX remains the better option for F(ab')2 subunit analysis.

With this AEX-MS method the Fc critical quality attributes can be performed without peptide mapping, saving time and reducing errors/and even more important reproducible results.

Literature

[1] Y. Yan, A. P. Liu, S. Wang, T. J. Daly, N. Li, Ultrasensitive Characterization of Charge Heterogeneity of Therapeutic Monoclonal Antibodies Using Strong Cation Exchange Chromatography Coupled to Native Mass Spectrometry, Anal. Chem. 2018, 90, 13013-20.

[2] A. P. Liu, Y. Yan, S. Wang, N. Li, Coupling Anion Exchange Chromatography with Native Mass Spectrometry for Charge Heterogeneity Characterization of Monoclonal Antibodies, Anal. Chem. 2022, 94, 6355–62.