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An Online Two-Dimensional Approach to Characterizing the Charge-Based Heterogeneity of Recombinant Monoclonal Antibodies Using a 2D-CEX–AEX–MS Workflow

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Introduction

Charge heterogeneity is an important product quality attribute for monoclonal antibodies because it can affect their stability, pharmacokinetics, and efficacy. Charge heterogeneity can arise from various factors, such as changes in the pH or temperature during the production process or enzymatic or chemical modifications of the protein. Modifications such as deamidation, glycation, cysteinylation, reduced disulfide bonds, and sialic acid contribute to the formation of acid variants. On the other hand, methionine oxidation, C-terminal lysine, succinimide, or glycine amidation and incomplete disulfide bonds contribute to the creation of basic variants of mAb.

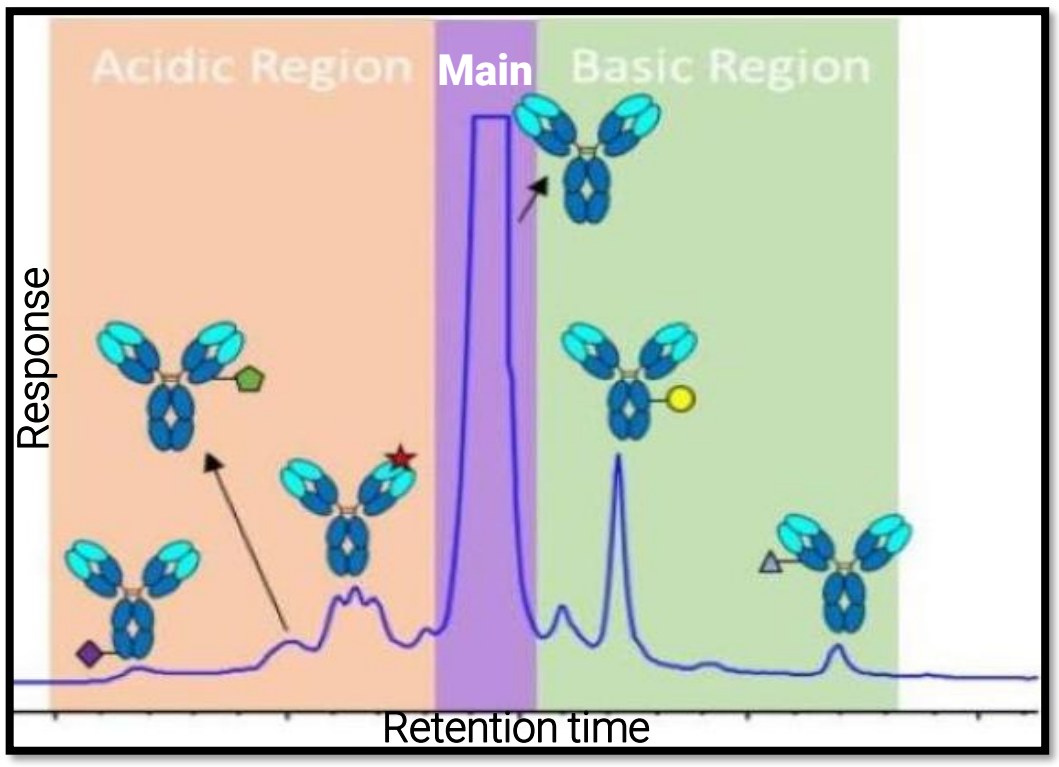


Fig 1 Graphical overview of Acidic and Basic charge variants of mAb by Ion Exchange Chromatography (IEX) or Capillary Electrophoresis.

Both CEX (Cation Exchange Chromatography) and AEX (Anion Exchange Chromatography) methods are widely used analytical tools for the characterisation of charge variants. In the present study, to resolve co-eluting variants of CEX and AEX chromatography, a volatile-salt-based mobile phase alongside the Agilent 1290 Infinity II 2D-LC system and Agilent 6545XT AdvanceBio LC/Q-TOF/MSMS workflow was described to separate the charge variants of mAbs using CEX chromatography in the first dimension and AEX chromatography coupled with MS in the second dimension for enhanced separation of the charge variants from the main peak (main species of the CEX profile).

Methodology

CEX-MS
- Development of CEX stand-alone methods on Agilent Bio SCX NP5 column using ammonium acetate buffer
- Hyphenating it with Mass Spectrometry

AEX-MS
- Development of AEX stand-alone methods using Agilent Bio SAX NP5 column using ammonium acetate buffer as mobile phase
- Hyphenating it with Mass Spectrometry

2D-CEX-AEX-MS
- Setting a ^{2}D platform with CEX in first dimension and AEX in second dimension
- Hyphenating it with Mass Spectrometry

Peptide Mapping
- Post-translational modifications that resulted in charge variants were confirmed through peptide mapping

Fig 2: Flow-chart representation of methodology

Method Parameters	CEX-MS	AEX-MS	2D CEX AEX-MS
Column	Agilent Bio SCX, NP5 column (4.6 mm × 250 mm, 5 µm)	Agilent Bio SAX, NP5 column (4.6 × 250 mm, 5 µm,	Both dimensions were interconnected through ▪ one 2-position/8-port ^{2}D valve ▪ two 6-position/14 -port valves ▪ one ASM capillary.
Mobile phase	▪ Mobile phase A: 15 mM ammonium acetate pH 6.8 ▪ Mobile phase B was 100 mM ammonium acetate pH 9.0	▪ Mobile phase A: 15 mM ammonium acetate (pH 9.5) ▪ Mobile phase B was 100 mM ammonium acetate (pH 4.5).	
Gradient	▪ mAb I and III 10-60%B ▪ mAb II 15-70% B	0-60 % linear gradient	
Flow rate	0.4 mL/min	0.4 mL/min	
Run time	30 minutes	30 minutes	

Table 1: Method Parameters

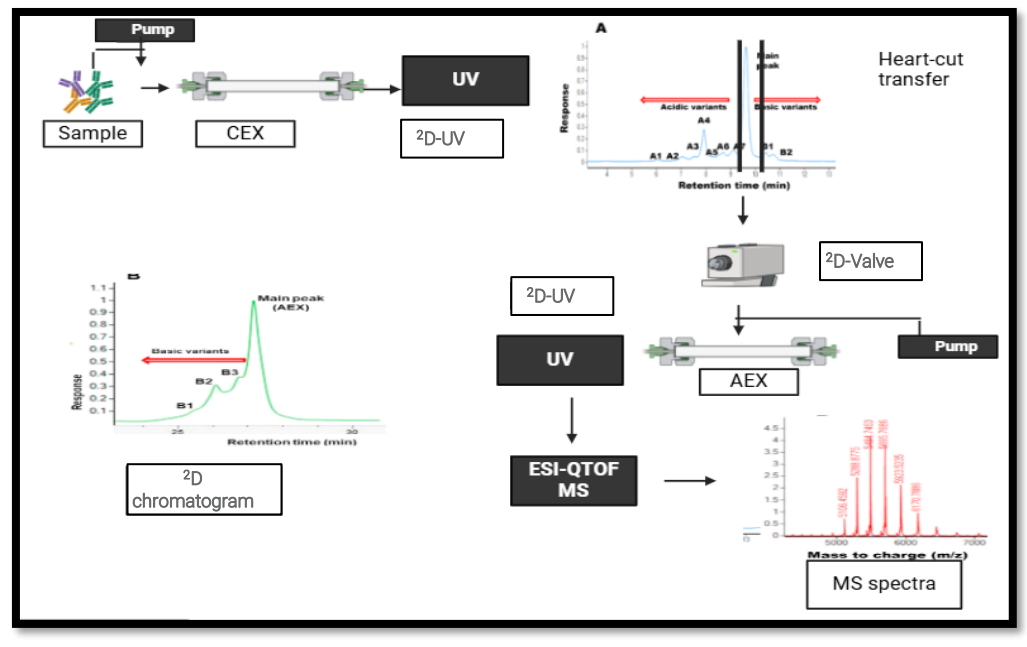
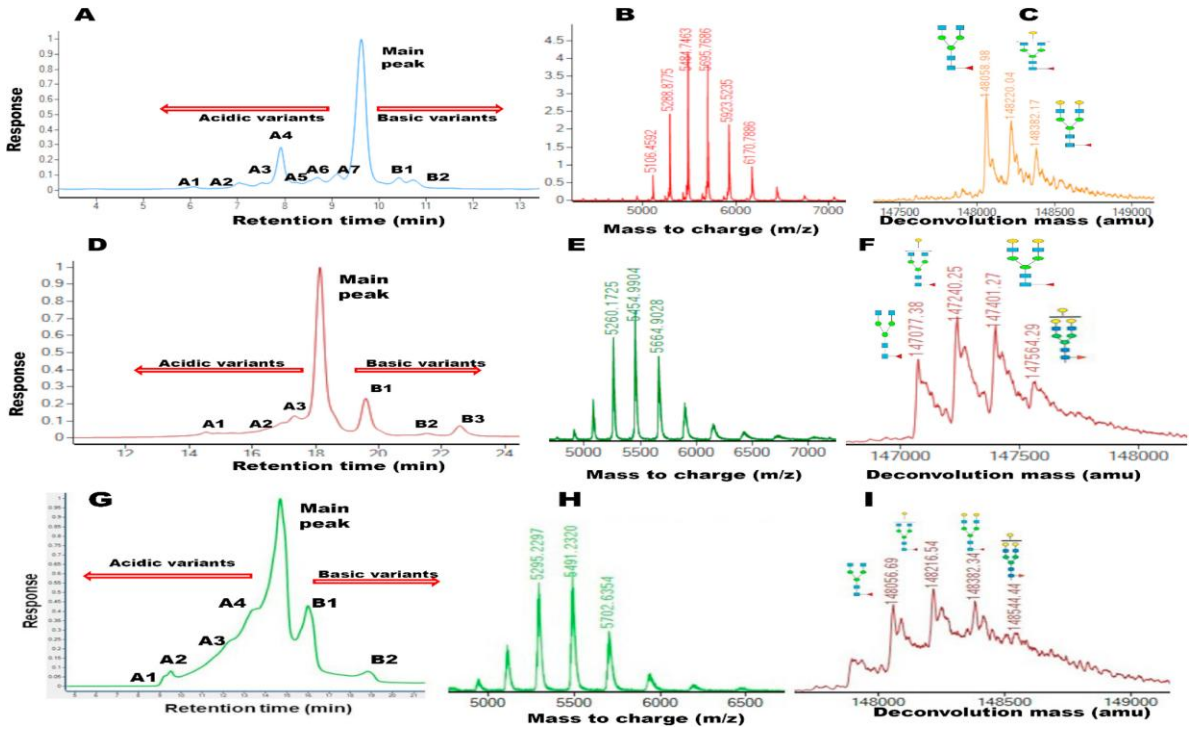


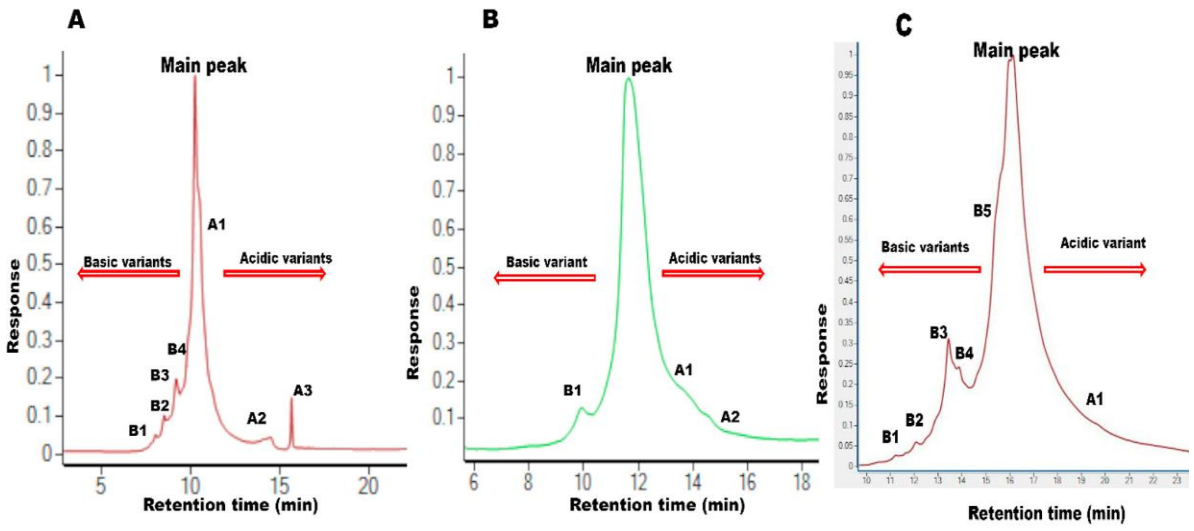
Fig 3: Graphical Representation of Methodology

Results and Discussion



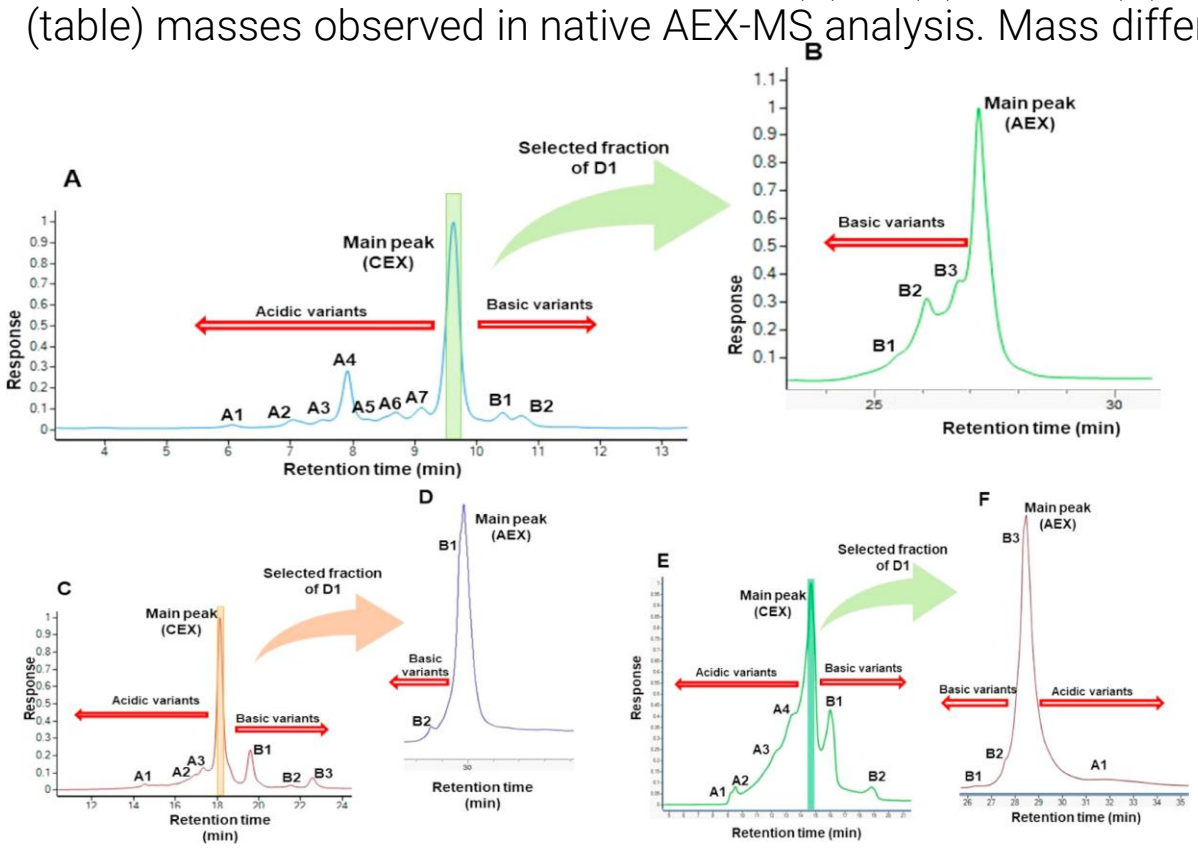
mAb I		
Variants	Mol. Wt (Da.)	Modification
A1	148065	7X deamidation
A2	148062	4X deamidation
A3	148061	3X Deamidation
A4	148060	2X Deamidation
A5	148060	Isomerization of Asp
A6	148226	Deamidation of G0F/G1F
A7	148059	1X Deamidation
M	148058	Unmodified(G0F/G0F)
B1	1480459	Isomerization of Asp
B2	148041	Pyroglutamic acid

Figure 4: CEX chromatogram and MS spectrum of mAb I, II and III and Deconvolution result summary of mAb I variants (table) masses observed in native CEX-MS analysis. Left panel: chromatogram; middle: MS spectra; left: deconvoluted spectra. Mass difference calculated from main peak (M)



mAb I		
Variants	Mol. Wt (Da.)	Modification
B1	147606	−2XGlcNAc+C-terminal amidation
B2	147854	−1XGlcNAc
B3	148059	Isomerization of Asp
B4	148058	Isomerization of Asp
M	148057	G0F/G0F(Unmodified)
A1	148059	2Xdeamidation
A2	148060	3X Deamidation
A3	148061	4X Deamidation

Figure 5: AEX chromatogram of mAb I (A), II (B) and III (C) and Deconvolution result summary of mAb I variants (table) masses observed in native AEX-MS analysis. Mass difference calculated from main peak (M)



mAb I		
Variants	Mol. Wt (Da.)	Modification
B1	147655	−2XGlcNAc
B2	147603	−2XGlcNAc+C-terminal amidation
B3	147855	−1XGlcNAc
M	148058	G0F/G0F

Figure 6: (A-B) CEX chromatogram of mAb I in the ^{1}D analysis (A) and selected peak fraction of ^{1}D analyzed in ^{2}D (B). (C-D) CEX chromatogram of mAb II in the ^{1}D analysis (C) and selected peak fraction of ^{1}D analyzed in ^{2}D (D). (E-F) CEX chromatogram of mAb III in the ^{1}D analysis (E) and selected peak fraction of ^{1}D analyzed in ^{2}D (F). Deconvolution results summary of mAb I variant (table) masses observed in 2D-CEX−AEX−MS analysis.

Results and Discussion

The volatile-salt-based methods offer several advantages over traditional nonvolatile buffer (phosphate buffer) IEX methods, such as minimizing artificial artifacts by avoiding the desalting step and in-depth characterization through MS. The second-dimension results showed that the 2D-CEX–AEX method identified more variants (13 for mAb I, nine for mAb II, and 11 for mAb III) than stand-alone CEX (10 for mAb I and seven each for mAb II and mAb III) or AEX (eight for mAb I, four for mAb II, and seven for mAb III).

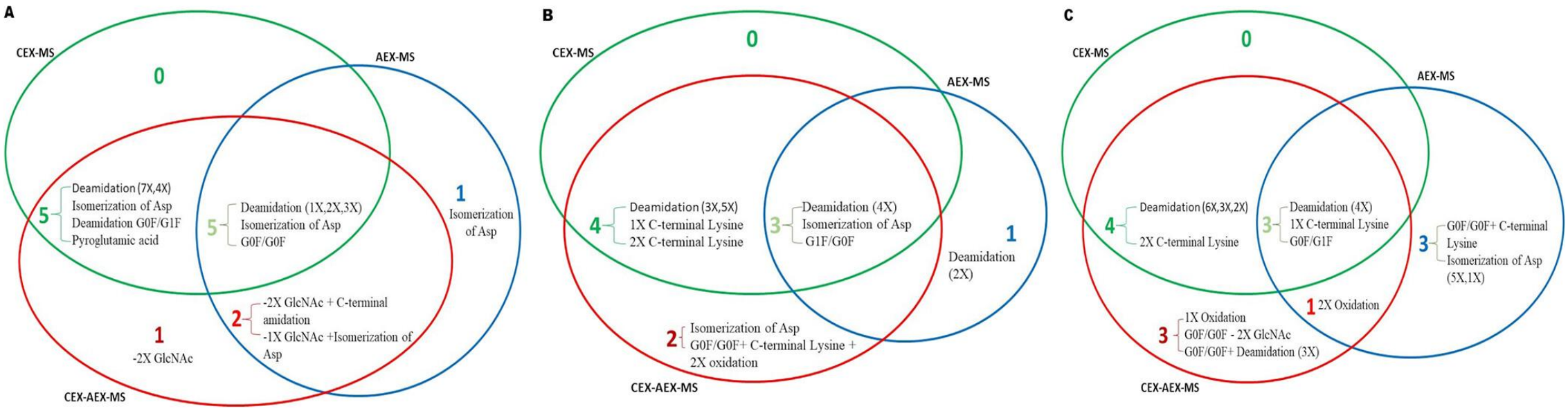


Figure 7. Venn Diagram showing charge variant distribution of mAb I , mAb II and mAb III. The green circle represents variants identified in the stand-alone CEX–MS method, the blue circle represents variants identified in the stand-alone AEX–MS method, and the red circle represents variants identified by the 2D-CEX–AEX–MS method.

Conclusions

The conventional CEX methods separate molecules based on their positive charge, but they are ineffective for analyzing monoclonal antibodies (mAbs) with near-neutral isoelectric points (pI). This limitation can be addressed using the AEX technique. The use of orthogonal methods can help to reveal subtle differences in the charge distribution or conformation of different variants of the mAb that cannot be detected by a single analytical method alone. In the presented ^{2}D method, the AEX method successfully resolved variants such as differences in glycosylation patterns, isomerization of Asp, and oxidation in the main peak, which were not separated in the ^{1}D method. By exploiting the subtle differences in charge, AEX–MS enabled the distinct isolation and characterization of these variants.

References

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