Characterization of Cetuximab using pH Gradient Cation Exchange and Microchip Electrophoresis Coupled to Native Orbitrap Mass Spectrometry

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Introduction

- **Charge variant analysis hyphenated to native Orbitrap mass spectrometry (CVA-MS) has been shown to be a powerful method for monitoring multiple product quality attributes (PQAs) of monoclonal antibodies (mAbs) including N-glycosylation, C-terminal lysine clipping, N-terminal pyroglutamate formation, deamidation, succinimide formation, proline amidation, glycation and low molecular weight species formation, using intact mass analysis [1,2].**
- **Application of CVA-MS for the characterisation of Cetuximab, a chimeric human-mouse mAb targeting epidermal growth factor receptor is presented. Cetuximab is a complex mAb, with two N-glycosylation sites present at asparagine N^{88} and N^{299} of each heavy chain, with C-terminal lysine variants also reported. These posttranslational modifications create a diverse and complex range of charge variant isoforms of Cetuximab.**
- **CVA-MS analysis performed using pH gradient elution with volatile buffers on a 2.1 × 50 mm Thermo Scientific MAbPac™ SCX-10 RS strong cation exchanger on a Thermo Scientific Vanquish™ Horizon UHPLC instrument hyphenated to a Thermo Scientific Q-Exactive™ Plus hybrid quadrupole Orbitrap mass spectrometer with extended mass Biopharma Option capabilities.**
- **Analysis also performed using the ZipChip® microchip electrophoresis separation platform hyphenated to Q-Exactive™ Plus Orbitrap MS demonstrating excellent comparability with CVA-MS for the charge variant profiling of Cetuximab.**

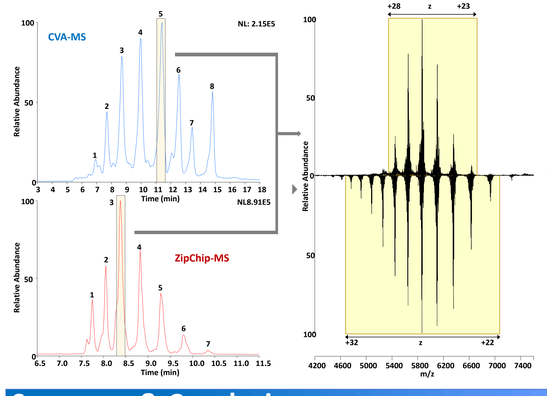
Results

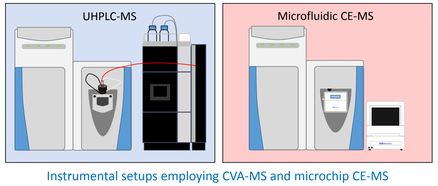
1. System Performance:

CVA-MS and ZipChip®-MS of Cetuximab revealed comparable separations of charge variants present. Both orthogonal separation mechanisms yielded complex charge variant separations with similar but not the same selectivity. Transfer into the Orbitrap resulted in informative native spectra with charge distributions of +23 to +28 for CVA peaks and +22 to +32 for the ZipChip®-MS arising from the different separation mechanisms. Deconvolution of total chromatogram/electropherogram resulted a complex spectrum containing a high number of spectral features.

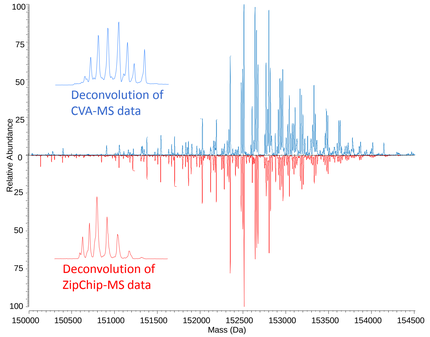
Comparable separations obtained using CVA-MS and ZipChip-MS, similar overall method times including re-equilibration.

Native spectra collected for the main peak present in the CVA chromatogram / ZipChip-MS electropherogram.





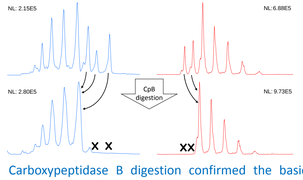
Instrumental setups employing CVA-MS and microchip CE-MS



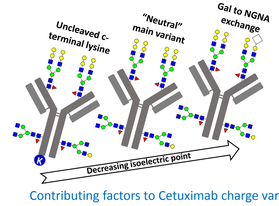
Complex spectra obtained following deconvolution resulting from glycan and C-terminal lysine distribution. Excellent comparability in spectral data obtained using CVA-MS or ZipChip-MS.

2. Orthogonal Selectivity:

CVA-MS and ZipChip®-MS provide orthogonal selectivity and directly reversed elution/migration order. Evidently, charge variant complexity is caused by various degrees of incomplete c-terminal lysine clipping and sialylation of Fab glycans. Several peaks do not only contain a single charge variant species but several which however exhibit the same net surface charge.

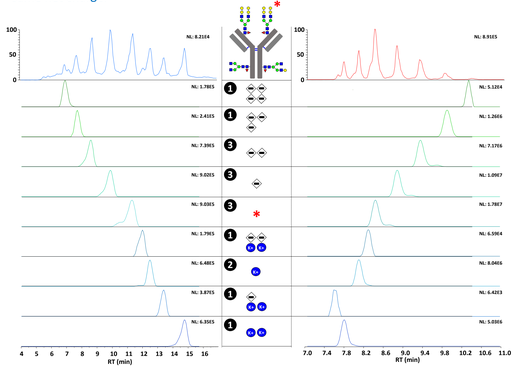


Carboxypeptidase B digestion confirmed the basic species arise from incomplete c-terminal lysine truncation.



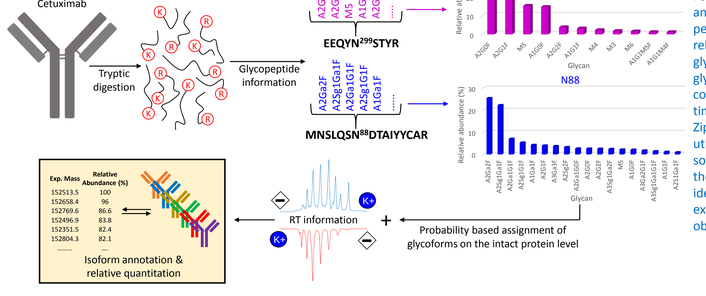
Contributing factors to Cetuximab charge variants.

Assignment of charge variant peaks to the charge variant species found. The red star represents the main species. Modifications impacting net charge are incomplete c-terminal lysine truncation and sialylation. White numbers in black circles represent the number of different combinations of these modifications found per peak resulting in the same net charge.

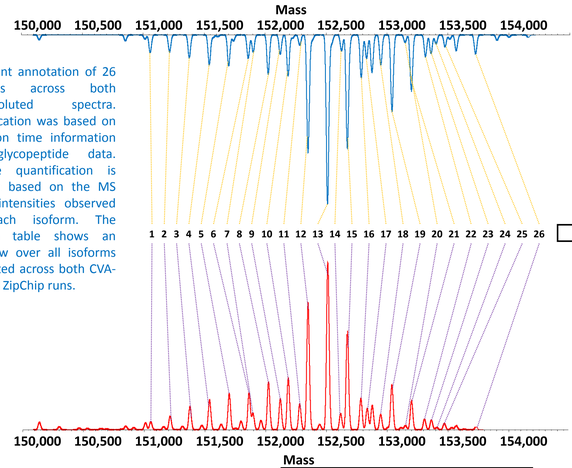


3. Proteoform annotation and relative quantification

Annotation was supported by peptide mapping and utilization of glycopeptide data using the MoFi software [3]. Glycoform assignment was hence probability based and expected degrees of c-terminal lysine and sialylation were deduced from retention/migration times.



Isoform annotation using the MoFi software. Foundation for glycoform annotation was a tryptic peptide map yielding relative abundances of glycans present on both glycosylation sites. Also considering retention time in CVA-MS or ZipChip-MS, this data was utilized by the MoFi software to determine the most probable identity for each experimental intact mass obtained.



Confident annotation of 26 isoforms across both deconvoluted spectra. Identification was based on retention time information and glycopeptide data. Relative quantification is feasible based on the MS signal intensities observed for each isoform. The bottom table shows an overview over all isoforms annotated across both CVA-MS and ZipChip runs.

Variant	K+	N299_1	N299_2	N88_1	N88_2
1	0	A2G0F	A2G0F	A2G0F	A2G0F
2	0	A2G0F	A2G0F	A2G1F	A2G0F
3	0	A2G0F	A2G0F	A2G1F	A2G1F
4	0	A2G0F	A2G0F	A2Ga1G1F	A2G0F
5	0	A2G0F	A2G0F	A2Ga2F	A2G0F
6	0	A2G0F	A2G0F	A2Ga2F	A2G1F
7	0	A1G0F	A1G1M5F	A2Ga2F	A1Ga1F
8	0	A2G0F	A2G1F	A2Ga2F	A2G1F
9	0	A2G0F	M5	A2Ga2F	A2Ga2F
10	0	A2G0F	A2G0F	A2Ga2F	A2Ga1G1F
11	0	A2G1F	M5	A2Ga2F	A2Ga2F
12	0	A2G0F	A2G0F	A2Ga2F	A2Ga2F
13	0	A2G0F	A2G1F	A2Ga2F	A2Ga2F
14	1	A2G0F	A2G0F	A2Ga2F	A2Sg1Ga1F
15	0	A2G1F	A2G1F	A2Ga2F	A2Ga2F
16	1	A2G0F	A2G1F	A2Ga2F	A2Sg1Ga1F
17	2	A2G1F	M5	A2Sg1Ga1F	A2Sg1Ga1F
18	1	A2G1F	A2G1F	A2Ga2F	A2Sg1Ga1F
19	0	A2G0F	A2G1F	A2Ga2F	A3Ga3F
20	1	A2G0F	A2G0F	A2Sg1Ga1F	A3Ga3F
21	0	A2G1F	A2G1F	A2Ga2F	A3Ga3F
22	1	A2G0F	A2G1F	A3Ga3F	A2Sg1Ga1F
23	2	A2G1F	M5	A2Sg1Ga1F	A3Sg1Ga2F
24	1	A2G1F	A2G2F	A2Sg2F	A2Sg1Ga1F
25	1	A2G1F	A2G1F	A2Sg1Ga1F	A3Ga3F
26	0	A2G1F	A2G1F	A3Ga3F	A3Ga3F

Summary & Conclusion

- **Both techniques are orthogonal, yet yield highly selective charge variant separations clearly distinguishing 8 peaks in case of CVA-MS and 7 peaks in case of ZipChip-MS. MS spectra obtained by both approaches are of excellent quality and highly similar.**
- **Both data sets show that Cetuximab charge variant heterogeneity is caused by various degrees of incomplete c-terminal lysine truncation and sialylation which is also confirmed via enzymatic digestion.**
- **Charge variant peaks are not characteristic for a specific number of acidic and basic modifications but rather for the net charge resulting by their combinations.**
- **Separation under native conditions *e.g.* using either CVA-MS or ZipChip®-MS, increases the dynamic range of native MS analysis of complex proteins such as mAbs. Provision of comparable, orthogonal data increases confidence of annotated proteoforms based on retention or migration behaviour arising from the presence of acidic or basic modifications on the mAb.**
- **CVA-MS using the Thermo Scientific MAbPac™ SCX-10 RS and 908 Devices ZipChip®-MS using the Native HR separation platform are highly comparable, providing excellent and orthogonal selectivity and high efficiency separation of mAb charge variants under native conditions.**

References

1. **F. Fuessl *et al.* Anal. Chem. 2018, *90*, 4669-4676.**
2. **F. Fuessl *et al.* mAbs, 2019, *11*, 116-128.**
3. **W. Skala *et al.* Anal. Chem. 2018, *90*, 5728-5736.**