# Microfluidic Capillary Electrophoresis - Native Orbitrap Mass Spectrometry to unravel top selling monoclonal antibodies heterogeneity

Sara Carillo[1], Florian Füssl[1], Craig Jakes[1], Ashley Bell[2], Erin A. Redman[2], Jonathan Bones[1,3]

[1]Characterisation and Comparability Laboratory, NIBRT. Foster Avenue, Mount Merrion, Blackrock, co. Dublin. Ireland
[2]908 Devices Inc., Boston, MA.
[3]School of Chemical and Bioprocess Engineering, University College Dublin, Belfield, Dublin 4

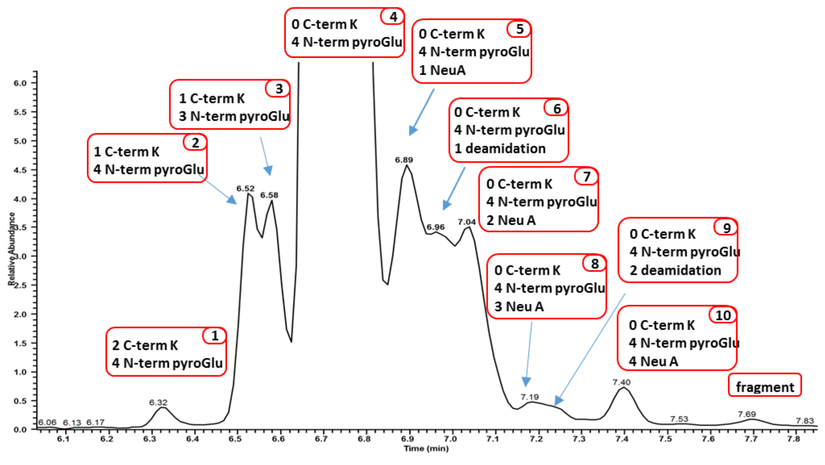
nibrt
National Institute for Bioprocessing Research and Training

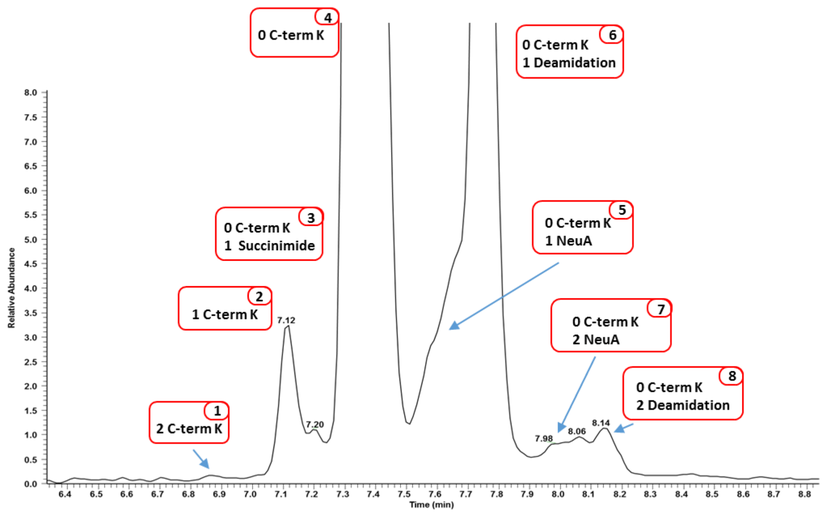
## Background

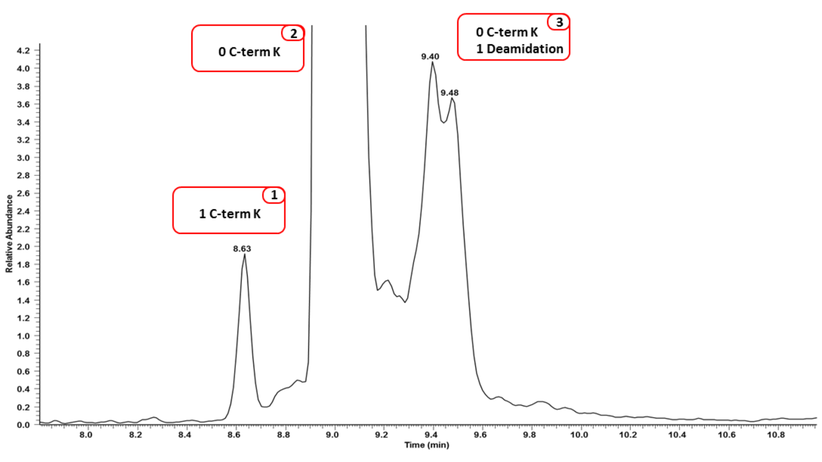
**The biopharmaceutical industry continues to develop mAb-based biotherapeutics for several application and diseases treatment. The complexity of these molecules creates a considerable challenge for the analytical technologies used to monitor the product quality attributes (PQAs) that need to be measured and controlled to guarantee safety and efficacy [1]. According to ICH guidelines, one of the features that needs to be monitored during biopharmaceutical development, and during approval and batch release post authorization, is the charge variant profile of a biotherapeutic. Standardized methods include the use of capillary electrophoresis using either zone or isoelectric focusing separation modes or the use of ion exchange liquid chromatography with UV detection (IEX-UV) [2]. The recent development of native mass spectrometry has facilitated the use of such separation techniques with Orbitrap mass spectrometry detection to obtain on-line MS identification of the charge variant proteoforms [3,4]. Native MS offers several advantages that include fast analysis and minimal sample preparation when combined with an upfront analytical separation, thus avoiding artificially-induced modification on the analyte.**

**We tested the performance of ZipChip™ platform hyphenated with a QExactive™ Plus Hybrid Quadrupole Orbitrap mass spectrometer using the extended mass range Biopharma option available on this instrument to perform native MS experiments on the charge variants separated using microchip electrophoresis. The drug products tested in this work are rituximab, bevacizumab and trastuzumab; microfluidic CE-ESI analysis showed improved in-depth characterization of the biotherapeutics, identifying with high mass accuracy up to 52 proteoforms and with fast analysis requiring minimal optimization of the experimental conditions and minimal sample preparation.**


## Results




### Rituximab

| Primary sequence modifications | N-Glycan | Experimental Average | Theoretical mass | Avg Delta mass (ppm) | Rel abundance (%) |
|---|---|---|---|---|---|
| 4x N-term PyroGlu | 1xA2G2F,1xA2G1F | 147818.09 | 147818.11 | 0.14 | 0.01 |
| 4x N-term PyroGlu | 2xA2G1F | 147654.165 | 147655.96 | 12.16 | 0.05 |
| 4x N-term PyroGlu | 2xA2G0F | 147331.04 | 147331.68 | 4.34 | 0.07 |
| 4x N-term PyroGlu | 1xA2G0F,1xA2G1F | 147490.78 | 147493.82 | 20.61 | 0.06 |
| 4x N-term PyroGlu, 1x C-term lysine loss | 1xA2G0F,1xA2G1F | 147363.849 | 147365.65 | 12.22 | 1.50 |
| 4x N-term PyroGlu, 1x C-term lysine loss | 2xA2G0F | 147201.9792 | 147203.51 | 10.40 | 1.10 |
| 4x N-term PyroGlu, 1x C-term lysine loss | 1xA2G0F,1xA2G2F | 147526.25 | 147527.79 | 10.44 | 1.04 |
| 4x N-term PyroGlu, 1x C-term lysine loss | 1xA2G1F,1xA2G2F | 147688.1146 | 147689.94 | 12.36 | 0.36 |
| 4x N-term PyroGlu, 1x C-term lysine loss | 2xA2G2F | 147850.25 | 147852.09 | 12.44 | 0.06 |
| 3x N-term PyroGlu, 2x C-term lysine loss | 1xA2G0F,1xA2G1F | 147251.5677 | 147254.51 | 19.98 | 1.61 |
| 3x N-term PyroGlu, 2x C-term lysine loss | 2xA2G1F | 147413.2396 | 147416.65 | 23.13 | 1.24 |
| 3x N-term PyroGlu, 2x C-term lysine loss | 2xA2G0F | 147089.5104 | 147092.37 | 19.44 | 1.06 |
| 3x N-term PyroGlu, 2x C-term lysine loss | 1xA2G1F,1xA2G2F | 147574.474 | 147578.79 | 29.25 | 0.50 |
| 3x N-term PyroGlu, 2x C-term lysine loss | 1xA2G2F,1xA2G2F | 147736.8438 | 147740.93 | 27.66 | 0.13 |
| 3x N-term PyroGlu, 2x C-term lysine loss | 1xA2G0F,1xA2G0 | 146944.099 | 146945.85 | 11.92 | 0.06 |
| 4x N-term PyroGlu, 2x C-term lysine loss | 1xA2G0F,1xA2G1F | 147236.3021 | 147237.48 | 8.00 | 25.14 |
| 4x N-term PyroGlu, 2x C-term lysine loss | 2xA2G1F | 147398.3698 | 147399.62 | 8.48 | 22.71 |
| 4x N-term PyroGlu, 2x C-term lysine loss | 2xA2G0F | 147075.375 | 147075.34 | 0.24 | 14.94 |
| 4x N-term PyroGlu, 2x C-term lysine loss | 1xA2G1F,1xA2G2F | 147559.9948 | 147561.76 | 11.96 | 10.35 |
| 4x N-term PyroGlu, 2x C-term lysine loss | 2xA2G2F | 147721.4635 | 147723.9 | 16.49 | 2.72 |
| 4x N-term PyroGlu, 2x C-term lysine loss | 1xA2G0,1xA2G0F | 146930.349 | 146929.19 | 7.89 | 0.81 |
| 4x N-term PyroGlu, 2x C-term lysine loss | 2xM5 | 146616.4063 | 146618.85 | 16.67 | 0.27 |
| 4x N-term PyroGlu, 2x C-term lysine loss | 1xA2G1F,1xA2S1G1F | 147851.5521 | 147853.02 | 9.93 | 1.95 |
| 4x N-term PyroGlu, 2x C-term lysine loss | 1xA2G1F,1xA2S1F | 147689.7656 | 147690.87 | 7.48 | 1.25 |
| 4x N-term PyroGlu, 2x C-term lysine loss | 1xA2G2F,1xA2S1G1F | 148013.7656 | 148015.16 | 9.42 | 0.93 |
| 4x N-term PyroGlu, 2x C-term lysine loss | 1xA1G1F,1xA2S1F | 147481.474 | 147487.67 | 42.01 | 0.18 |
| 4x N-term PyroGlu, 2x C-term lysine loss | 1xA1G0F,1xA2S1F | 147324.3594 | 147325.53 | 7.95 | 0.10 |
| 4x N-term PyroGlu, 2x C-term lysine loss | 2xA2G0F,1xA2S1F | 147529.3438 | 147528.72 | 4.23 | 0.10 |
| 4x N-term PyroGlu, 2x C-term lysine loss, 1x Deamidation | 1xA2G0F,1xA2G1F | 147237.2031 | 147238.49 | 8.74 | 1.68 |
| 4x N-term PyroGlu, 2x C-term lysine loss, 1x Deamidation | 2xA2G1F | 147398.9427 | 147400.63 | 11.45 | 1.54 |
| 4x N-term PyroGlu, 2x C-term lysine loss, 1x Deamidation | 2xA2G0F | 147074.5729 | 147076.35 | 12.08 | 0.93 |
| 4x N-term PyroGlu, 2x C-term lysine loss, 1x Deamidation | 1xA2G1F,1xA2G2F | 147561.3854 | 147562.77 | 9.38 | 0.67 |
| 4x N-term PyroGlu, 2x C-term lysine loss, 1x Deamidation | 1xA2G0,1xA2G0F | 146931.4271 | 146930.2 | 8.35 | 0.05 |
| 4x N-term PyroGlu, 2x C-term lysine loss | 1xA2G1F,1xA2S2F | 148142.1719 | 148144.27 | 14.16 | 1.53 |
| 4x N-term PyroGlu, 2x C-term lysine loss | 1xA2G2F,1xA2S2F | 148304.9531 | 148306.41 | 9.82 | 1.06 |
| 4x N-term PyroGlu, 2x C-term lysine loss | 1xA2G0F,1xA2S2F | 147980.3438 | 147982.12 | 12.00 | 0.43 |
| 4x N-term PyroGlu, 2x C-term lysine loss, 2x Deamidation | 1xA2G0F,1xA2G1F | 147236.6615 | 147239.5 | 19.28 | 0.57 |
| 4x N-term PyroGlu, 2x C-term lysine loss, 2x Deamidation | 2xA2G1F | 147398.7552 | 147401.64 | 19.57 | 0.54 |
| 4x N-term PyroGlu, 2x C-term lysine loss, 2x Deamidation | 2xA2G0F | 147074.0938 | 147077.36 | 22.21 | 0.30 |
| 4x N-term PyroGlu, 2x C-term lysine loss | 1xA2S1G1F,1xA2S2F | 148596.3906 | 148597.68 | 8.68 | 0.17 |
| 4x N-term PyroGlu, 2x C-term lysine loss | 2xA2S2F | 148886.7552 | 148888.94 | 14.67 | 0.24 |
| Fragment | 1xA2G0F,1xA2G1F | 100076.35 | 100075.52 | 8.27 | |
| Fragment | 2xA2G1F | 100238.2318 | 100237.58 | 6.49 | |
| Fragment | 2xA2G0F | 99912.57 | 99913.46 | 8.92 | |
| Fragment | 1xA2G1F,1xA2G2F | 100402.03 | 100399.63 | 23.90 | |

* Only one N-glycan pair is indicated where it is not possible to determine the exact distribution of N-glycans between the two Fc regions. As an example 2xA2G1F could potentially correspond to 1xA2G0F, 1xA2G2F.

### Trastuzumab

| Primary sequence modifications | N-Glycan | Experimental Average | Theoretical mass | Avg Delta mass (ppm) | Rel abundance (%) |
|---|---|---|---|---|---|
| None | 2xA2G0F | 148316.02 | 148312.90 | 21.01 | 0.02 |
| 1x C-term lysine loss | 2xA2G0F | 148184.95 | 148184.73 | 1.47 | 0.67 |
| 1x C-term lysine loss | 1xA2G0F,1xA2G1F | 148346.44 | 148346.87 | 2.92 | 0.56 |
| 1x C-term lysine loss | 2xA2G1F | 148506.24 | 148509.01 | 18.62 | 0.31 |
| 1x C-term lysine loss | 2xM5 | 147725.38 | 147728.24 | 19.39 | 0.31 |
| 1x C-term lysine loss | 1xA2G1F,1xA2G2F | 148668.45 | 148671.15 | 18.17 | 0.09 |
| 1x C-term lysine loss | 1xA1G0F,1xA2G0 | 147834.33 | 147835.39 | 7.18 | 0.03 |
| 1x C-term lysine loss | 2xA2G0 | 147888.26 | 147892.44 | 28.28 | 0.12 |
| 1x C-term lysine loss | 1xA1G1,1xA2G0F | 147991.89 | 147997.53 | 38.10 | 0.03 |
| 1x C-term lysine loss | 1xA2G2F,1xM5 | 148279.81 | 148280.77 | 6.46 | 0.02 |
| 1x C-term lysine loss | 1xA2G0F,1xA2G0 | 148039.22 | 148038.59 | 4.25 | 0.33 |
| 2x C-term lysine loss, 1x Succinimide | 1xA2G0F,1xA2G1F | 148199.72 | 148201.67 | 13.17 | 0.40 |
| 2x C-term lysine loss, 1x Succinimide | 2xA2G0F | 148038.91 | 148039.53 | 4.21 | 0.35 |
| 2x C-term lysine loss, 1x Succinimide | 1xA2G0F,1xA2G2F | 148364.41 | 148363.81 | 4.07 | 0.18 |
| 2x C-term lysine loss, 1x Succinimide | 1xA2G0,1xA2G0F | 147889.60 | 147893.39 | 25.62 | 0.05 |
| 2x C-term lysine loss, 1x Succinimide | 1xA2G1F,1xA2G2F | 148520.28 | 148525.95 | 38.17 | 0.15 |
| 2x C-term lysine loss, 1x Succinimide | 1xA2G1,1xA2G2F | 148374.45 | 148379.81 | 36.10 | 0.03 |
| 2x C-term lysine loss | 2xA2G0F | 148055.76 | 148056.56 | 5.44 | 21.38 |
| 2x C-term lysine loss | 1xA2G0F,1xA2G1F | 148217.02 | 148218.70 | 11.33 | 24.01 |
| 2x C-term lysine loss | 2xA2G1F | 148378.74 | 148380.84 | 14.16 | 15.13 |
| 2x C-term lysine loss | 1xA2G1F,1xA2G2F | 148540.23 | 148542.98 | 18.48 | 5.56 |
| 2x C-term lysine loss | 1xA2G0,1xA2G0F | 147909.47 | 147910.42 | 6.43 | 4.06 |
| 2x C-term lysine loss | 2xA2G2F | 148702.34 | 148705.12 | 18.70 | 1.31 |
| 2x C-term lysine loss | 1xA2G1F,1xA2S1G0F | 148670.32 | 148672.09 | 11.89 | 1.03 |
| 2x C-term lysine loss | 1xA2G1F,1xA2S1G1F | 148832.09 | 148834.24 | 14.46 | 0.99 |
| 2x C-term lysine loss | 1xA1G1F,1xA2S1G0F | 148467.88 | 148468.89 | 6.80 | 0.41 |
| 2x C-term lysine loss | 1xA2G2F,1xA2S1G1F | 148993.44 | 148996.38 | 19.71 | 0.38 |
| 2x C-term lysine loss | 1xA2G0F,1xA2S1G0F | 148511.17 | 148509.95 | 8.23 | 0.35 |
| 2x C-term lysine loss | 1xA1G0F,1xA2S1G0F | 148306.45 | 148306.75 | 2.00 | 0.56 |
| 2x C-term lysine loss | 1xA1G0,1xA2S1G0F | 148159.23 | 148160.60 | 9.22 | 0.08 |
| 2x C-term lysine loss, 1x Deamidation | 1xA2G0F,1xA2G1F | 148217.74 | 148219.71 | 13.29 | 6.44 |
| 2x C-term lysine loss, 1x Deamidation | 2xA2G0F | 148056.78 | 148057.57 | 5.33 | 4.76 |
| 2x C-term lysine loss, 1x Deamidation | 2xA2G1F | 148379.34 | 148381.85 | 16.89 | 4.53 |
| 2x C-term lysine loss, 1x Deamidation | 1xA2G1F,1xA2G2F | 148541.58 | 148543.99 | 16.24 | 1.82 |
| 2x C-term lysine loss, 1x Deamidation | 1xA2G0,1xA2G0F | 147910.81 | 147911.43 | 4.21 | 0.82 |
| 2x C-term lysine loss, 1x Deamidation | 2xA2G2F | 148702.74 | 148706.13 | 22.80 | 0.43 |
| 2x C-term lysine loss, 1x Deamidation | 1xA2G1F,1xA2S1G0F | 148670.81 | 148673.10 | 15.39 | 0.19 |
| 2x C-term lysine loss, 1x Deamidation | 1xA2G1F,1xA2S1G1F | 148832.21 | 148835.25 | 20.44 | 0.18 |
| 2x C-term lysine loss, 1x Deamidation | 1xA2G0F,1xA2S1G0F | 148511.91 | 148510.96 | 6.37 | 0.06 |
| 2x C-term lysine loss, 1x Deamidation | 1xA1G0F,1xA2S1G0F | 148307.65 | 148307.76 | 0.77 | 0.07 |
| 2x C-term lysine loss, 1x Deamidation | 1xA2G2F,1xA2S1G1F | 148993.91 | 148997.39 | 23.35 | 0.07 |
| 2x C-term lysine loss, 1x Deamidation | 1xA1G0F,1xA2S1G1F | 148464.70 | 148469.90 | 35.00 | 0.03 |
| 2x C-term lysine loss, 1x Deamidation | 1xA1G1F,1xA2S1G1F | 148627.22 | 148632.05 | 32.50 | 0.32 |
| 2x C-term lysine loss, 2x Deamidation | 1xA2G0F,1xA2G1F | 148218.47 | 148220.72 | 15.19 | 0.44 |
| 2x C-term lysine loss, 2x Deamidation | 2xA2G1F | 148380.24 | 148382.86 | 17.62 | 0.36 |
| 2x C-term lysine loss, 2x Deamidation | 2xA2G0F | 148057.54 | 148058.58 | 7.01 | 0.29 |
| 2x C-term lysine loss, 2x Deamidation | 1xA2G1F,1xA2G2F | 148541.77 | 148545.00 | 21.74 | 0.14 |
| 2x C-term lysine loss, 2x Deamidation | 2xA2G2F | 148706.16 | 148707.14 | 6.62 | 0.01 |
| 2x C-term lysine loss, 2x Deamidation | 1xA2G0,1xA2G0F | 147910.07 | 147912.44 | 16.04 | 0.05 |
| 2x C-term lysine loss, 3x Deamidation | 2xA2G0F | 148057.61 | 148059.59 | 13.38 | 0.02 |
| 2x C-term lysine loss, 3x Deamidation | 1xA2G0F,1xA2G1F | 148218.14 | 148221.73 | 24.22 | 0.03 |
| 2x C-term lysine loss, 3x Deamidation | 2xA2G1F | 148380.89 | 148383.87 | 20.08 | 0.02 |

### Bevacizumab

| Primary sequence modifications | N-Glycan | Experimental Average | Theoretical mass | Avg Delta mass (ppm) | Rel abundance (%) |
|---|---|---|---|---|---|
| 1x C-term lysine loss | 2xA2G0F | 149323.29 | 149325.73 | 16.33 | 1.80 |
| 1x C-term lysine loss | 1xA2G0F,1xA2G1F | 149485.27 | 149487.87 | 17.42 | 0.71 |
| 1x C-term lysine loss | 1xA2G0F,1xA2G2F | 149646.73 | 149650.01 | 21.92 | 0.22 |
| 1x C-term lysine loss | 1xA1G0F,1xA2G0F | 149120.55 | 149122.54 | 13.37 | 0.15 |
| 1x C-term lysine loss | 1xA2G2F,1xM5 | 149420.20 | 149421.77 | 10.49 | 0.03 |
| 1x C-term lysine loss | 1xA2G0,1xA2G0F | 149182.38 | 149179.59 | 18.67 | 0.12 |
| 1x C-term lysine loss | 1xA2G0F | 147876.70 | 147880.40 | 25.00 | 0.03 |
| 2x C-term lysine loss | 2xA2G0F | 149195.23 | 149197.56 | 15.62 | 57.36 |
| 2x C-term lysine loss | 1xA2G0F,1xA2G1F | 149357.63 | 149359.70 | 13.89 | 19.78 |
| 2x C-term lysine loss | 1xA2G0F,1xA2G2F | 149519.02 | 149521.84 | 18.89 | 4.64 |
| 2x C-term lysine loss | 1xA1G0F,1xA2G0F | 148991.03 | 148994.36 | 22.38 | 3.68 |
| 2x C-term lysine loss | 1xA2G0F | 147750.45 | 147752.22 | 11.99 | 3.30 |
| 2x C-term lysine loss | 1xA2G0,1xA2G0F | 149050.11 | 149051.42 | 8.79 | 0.92 |
| 2x C-term lysine loss, 1 Deamidation | 2xA2G0F | 149197.31 | 149198.57 | 8.43 | 4.27 |
| 2x C-term lysine loss, 1 Deamidation | 1xA2G0F,1xA2G1F | 149358.70 | 149360.71 | 13.44 | 1.82 |
| 2x C-term lysine loss, 1 Deamidation | 2xA2G1F | 149520.56 | 149522.85 | 15.30 | 0.59 |
| 2x C-term lysine loss, 1 Deamidation | 1xA2G0F | 147751.61 | 147753.23 | 10.93 | 0.36 |
| 2x C-term lysine loss, 1 Deamidation | 1xA1G0F,1xA2G0F | 148992.10 | 148995.37 | 21.95 | 0.24 |

## Materials & Methods

100 ug of mAb were buffer-exchanged with ZipChip Native Diluent in 0.5 mL spin-filters with 10KDa MWCO and concentrated to 0.5 mg/mL..

CE separation was carried on a ZipChip™ platform using an HRN microchip and native BGE. CE separation was carried for 15 minutes using an injection of 2 nL and a field strength of 500 V. The system was hyphenated with a QExactive Plus hybrid quadrupole Orbitrap mass spectrometer with extended mass Biopharma Option (Thermo Scientific).

| MS Settings | |
|---|---|
| HMR mode | On |
| Resolution | 35,000 |
| Microscan | 5 |
| Max Injection time | 20 ms |
| In-source CID | 150 |
| S-lens | 200 |

| Deconvolution settings | |
|---|---|
| Algorithm | Respect™ |
| Output Mass range | 146,000-150,000 |
| Charge state rage | 20-50 |
| Min. Adjacent charge states | 5 |
| Deconvolution mass tolerance | 15 ppm |
| Input mass range | 4000-8000 m/z |

**The charge variant analysis of 3 blockbuster biotherapeutics was performed on a microfluidic CE-ESI platform consisting of a ZipChip device, equipped with an HRN microchip, hyphenated with a QExactive Plus Orbitrap mass spectrometer. For rituximab drug product 45 proteoforms were identified, including acidic variants with up to 4 sialic acids and a mAb fragment constituted by a complete Fc region and only one Fab arm. This fragmentation has already been reported in the literature and could be caused by hydrolysis, β-elimination or by specific enzymatic cleavage of Cathepsin L, a protein often found as host cell protein in biotherapeutics formulations. Trastuzumab drug product revealed a major sample complexity, with 52 proteoforms identified and several isobaric variants having deamidation at different sites, thus migrating in different ways in the electropherogram. The same is visible in bevacizumab drug product, where 18 proteoforms were identified, but more isobaric variants are present, like the two deamidated species at rt 9.40 and 9.48.**

## Conclusions

**Biopharmaceuticals characterisation is becoming more demanding in terms of in-depth of the analysis, robustness and accuracy, possibly together with minimal sample preparation and fast analysis. Data obtained showed improved sensitivity with respect to standard native analysis performed by SEC-MS or direct infusion, analyzing amount of sample as low as 1ng. Robustness of the platform was proved by reproducible separation and quantitation, estimated through the deconvoluted peaks relative abundances in triplicate. As well, access to extended mass range instrument, allowed the acquisition of native spectra without sacrificing resolution; data analysis showed the determination of protein ID with high mass accuracy (Δ values rarely exceeding 30 ppm). All the data presented here-in demonstrate the applicability of microfluidic CE-ESI analysis for biopharmaceuticals characterisation in crucial stages of biomanufacturing like early stage development and clones evaluation as well as biosimilars comparability.**

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