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High-Performance Native Separation of mAb Proteoforms by CZE-MS under Native and Denaturing nanoESI Conditions for Ultrasensitive Charge Variant Characterization

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abbvie

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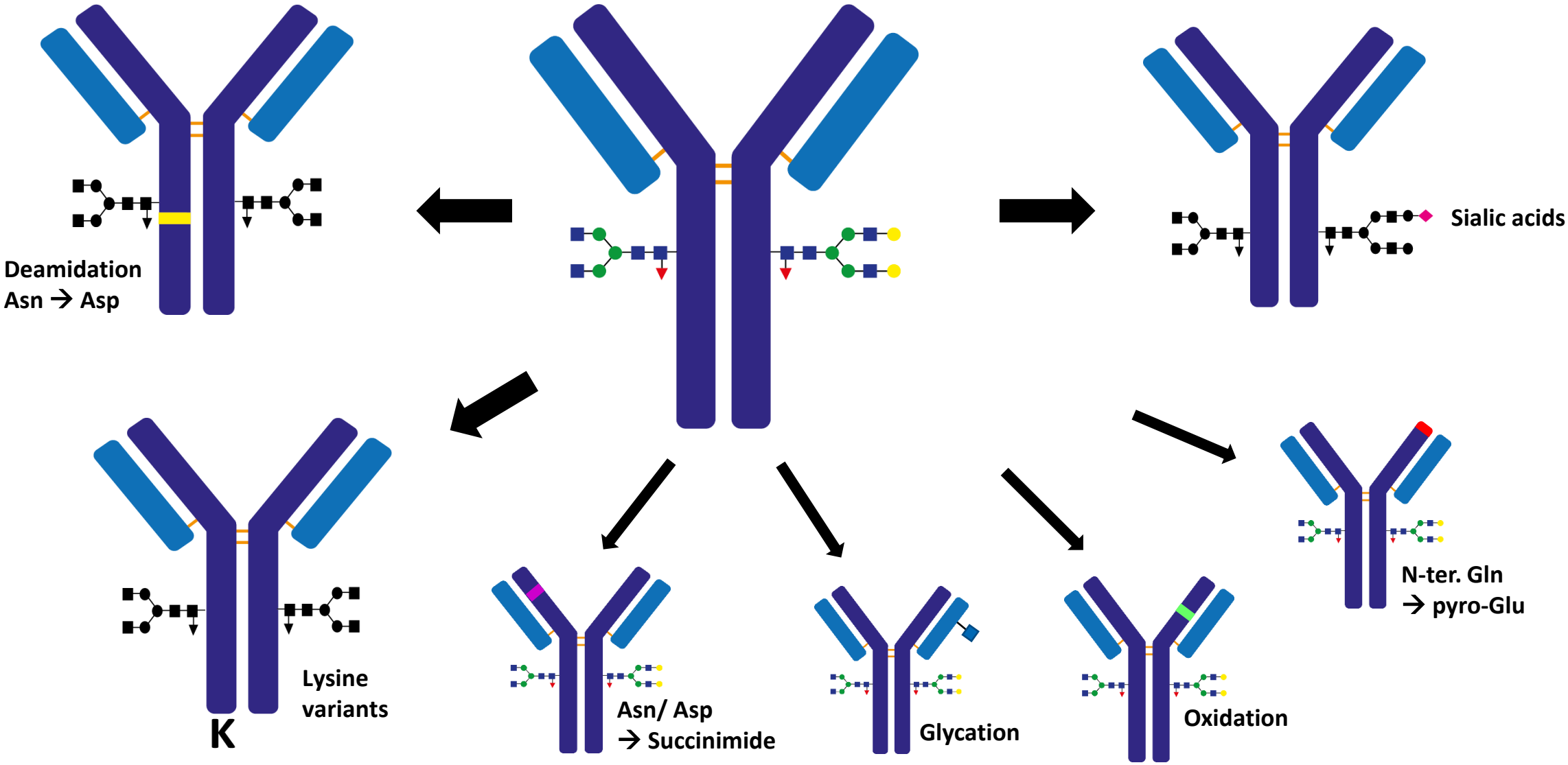
LC/CE-MS AKCN

Therapeutic antibodies – a mixture of proteoforms



Some **charge variants** might influence **potency** or **safety** of the biopharmaceutical → should be **monitored**

Separation of mAb charge variants by CZE

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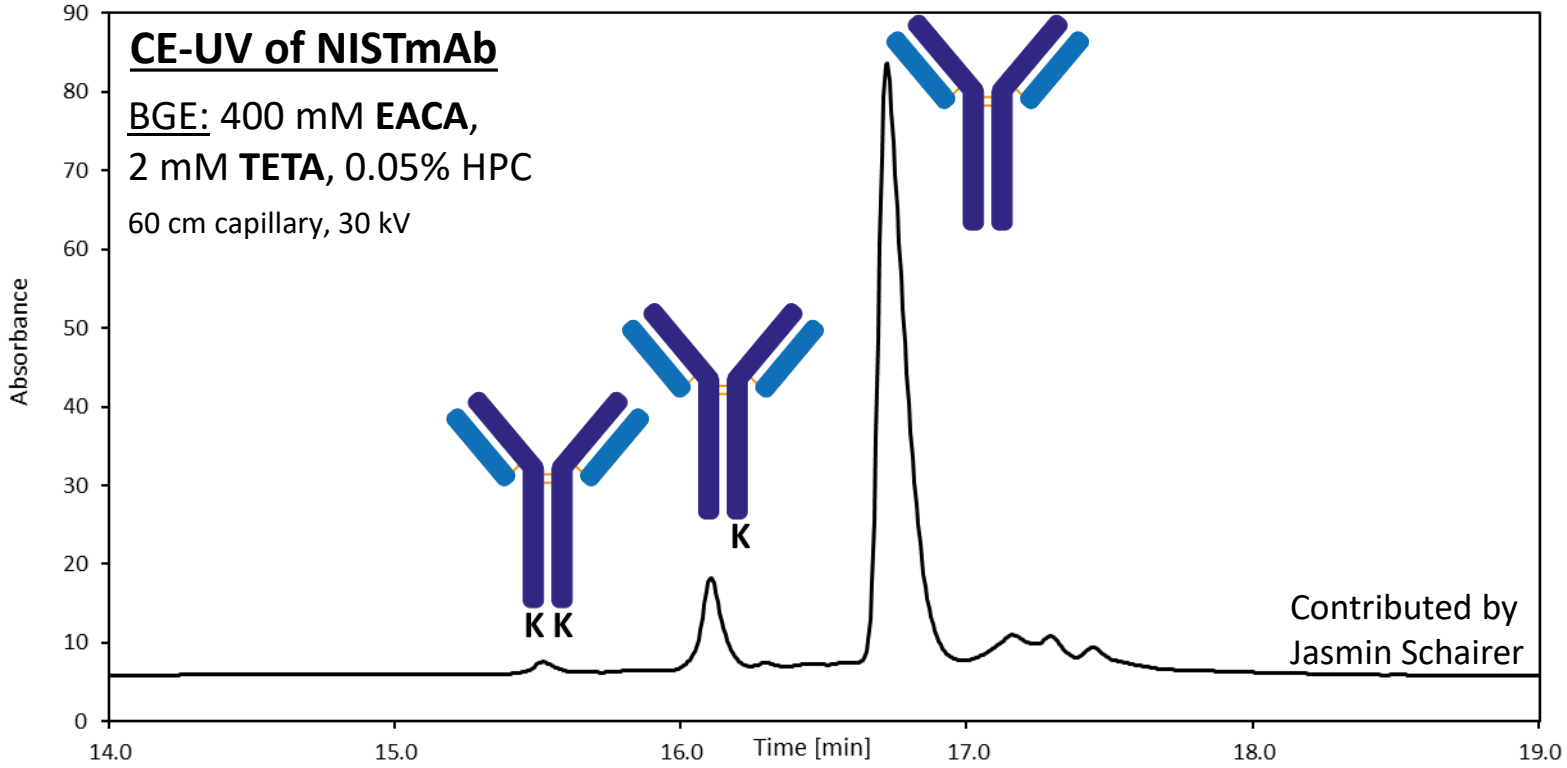
- Commonly applied **CZE-UV** method for charge variant separation involves **EACA** and **TETA** in the electrolyte:

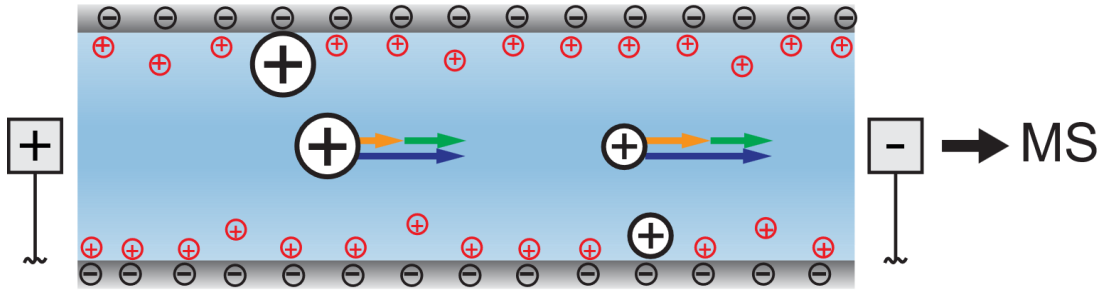


→ Very **good separation** but **not MS compatible** (non-volatile electrolyte)

How can we do CZE-**MS** analysis of mAb charge variants?

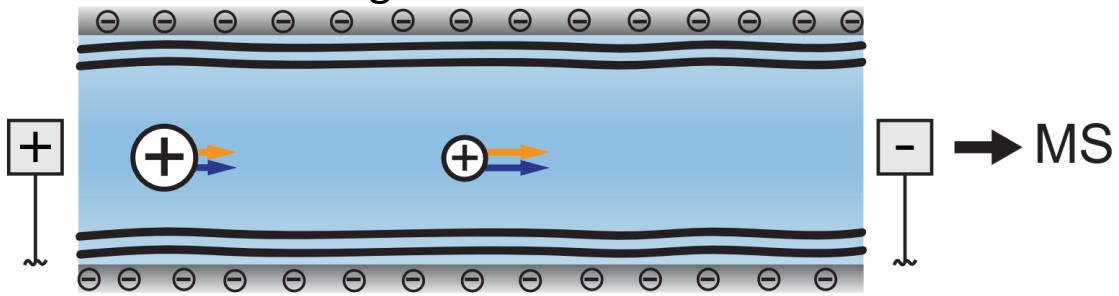
Introduction to coatings in CE

- **Uncoated**:



→ Decreases, broadens, or even eliminates analyte signals

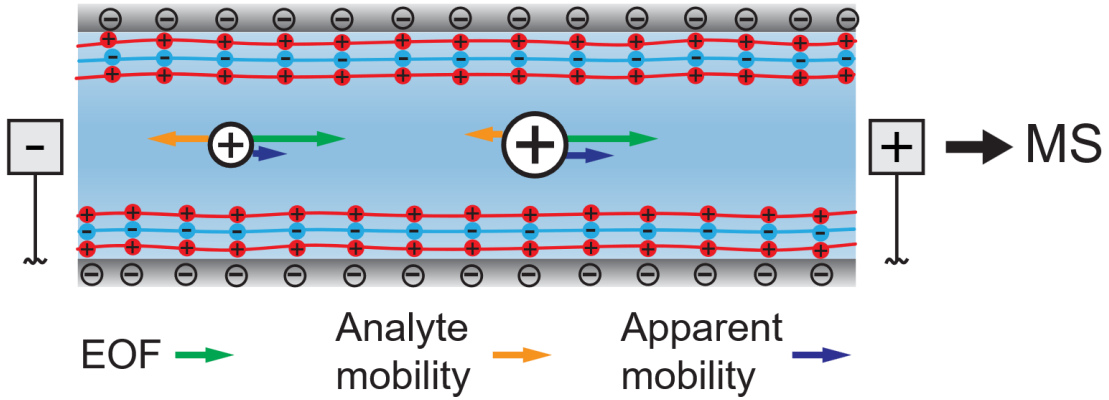
- **Neutral** coating:



→ Reduction of protein interactions with capillary wall

→ Supression of the EOF

- **Cationic** coating:

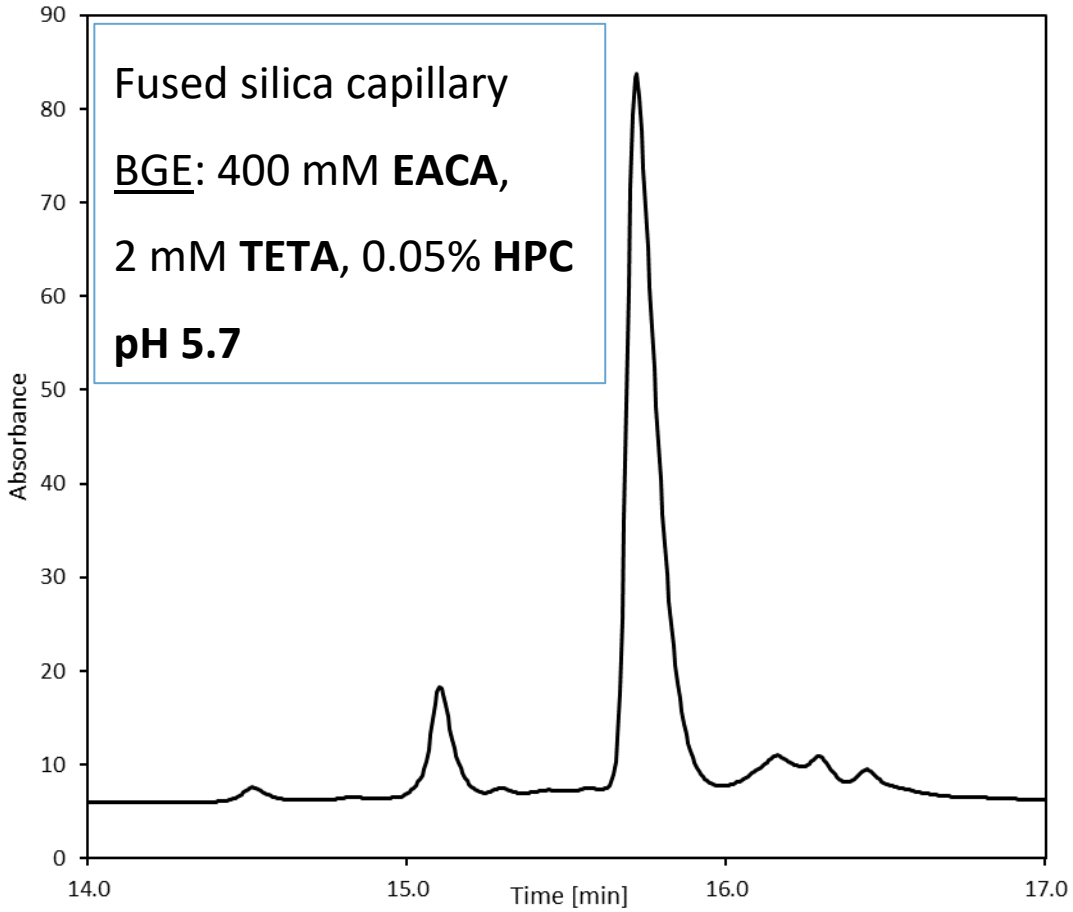


→ Reduction of protein interactions with capillary wall

→ Reversed EOF

* Separation of protein mix (Lysozym, RNase A, Myoglobin); 30 kV; BGE: 2 M Hac; 60 cm capillary; top: fused silica capillary; bottom: HPC coated capillary

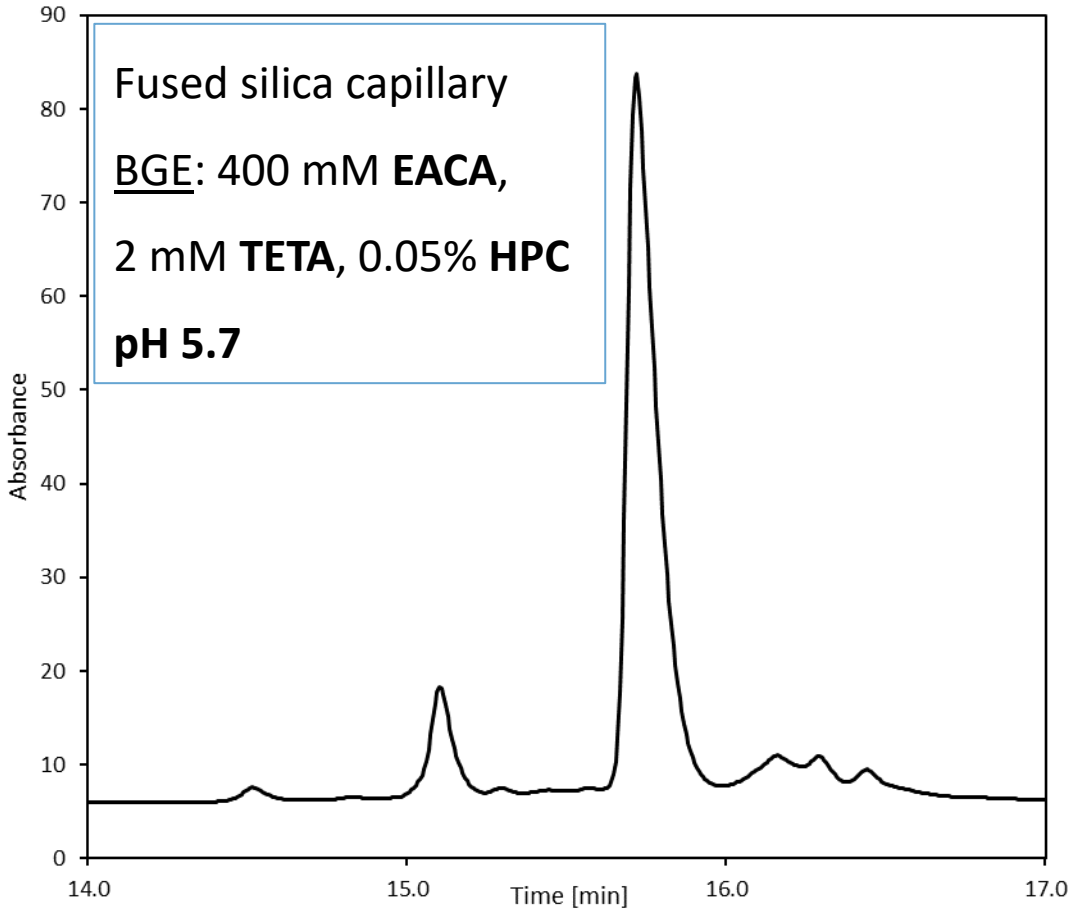
CE-UV of NISTmAb using different electrolytes



→ Efficient separation of charge variants ✓

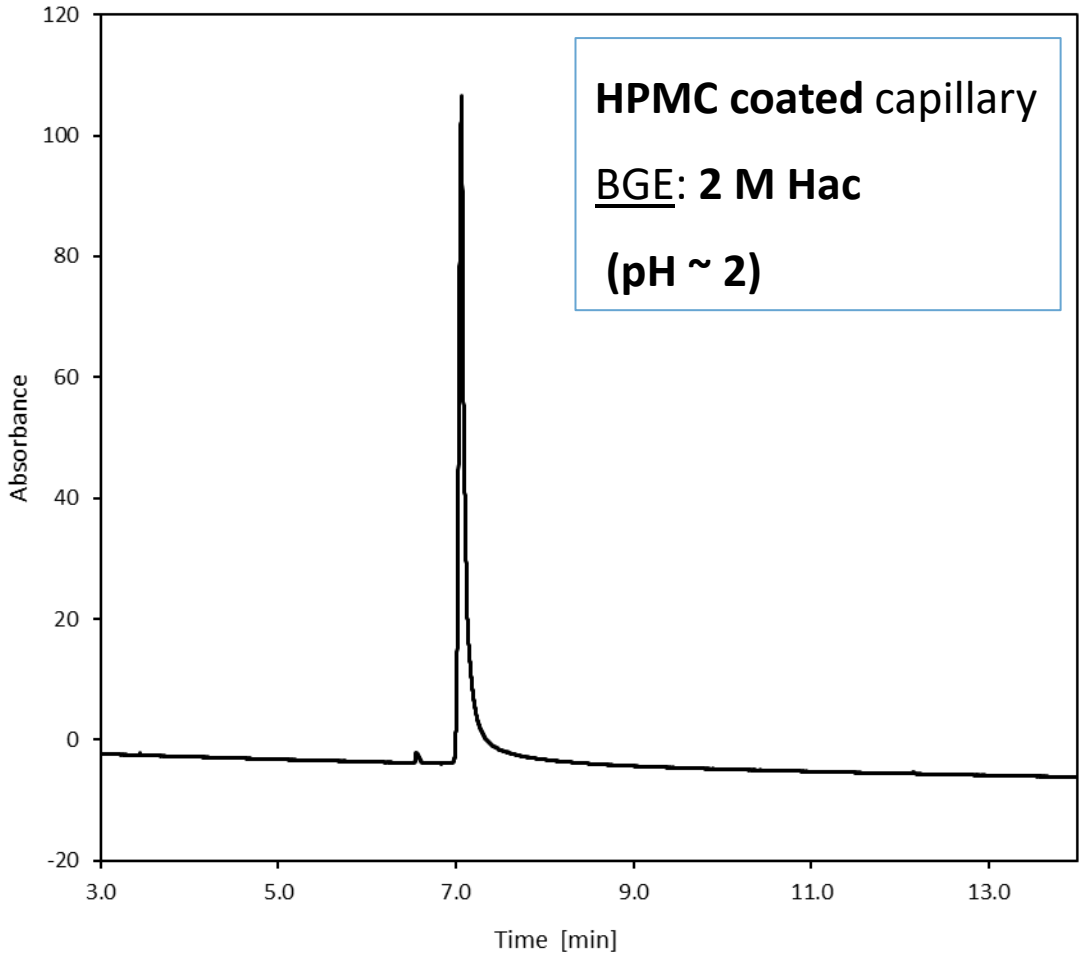
→ But: **Not** MS compatible ✗

CE-UV of NISTmAb using different electrolytes



→ Efficient separation of charge variants ✓

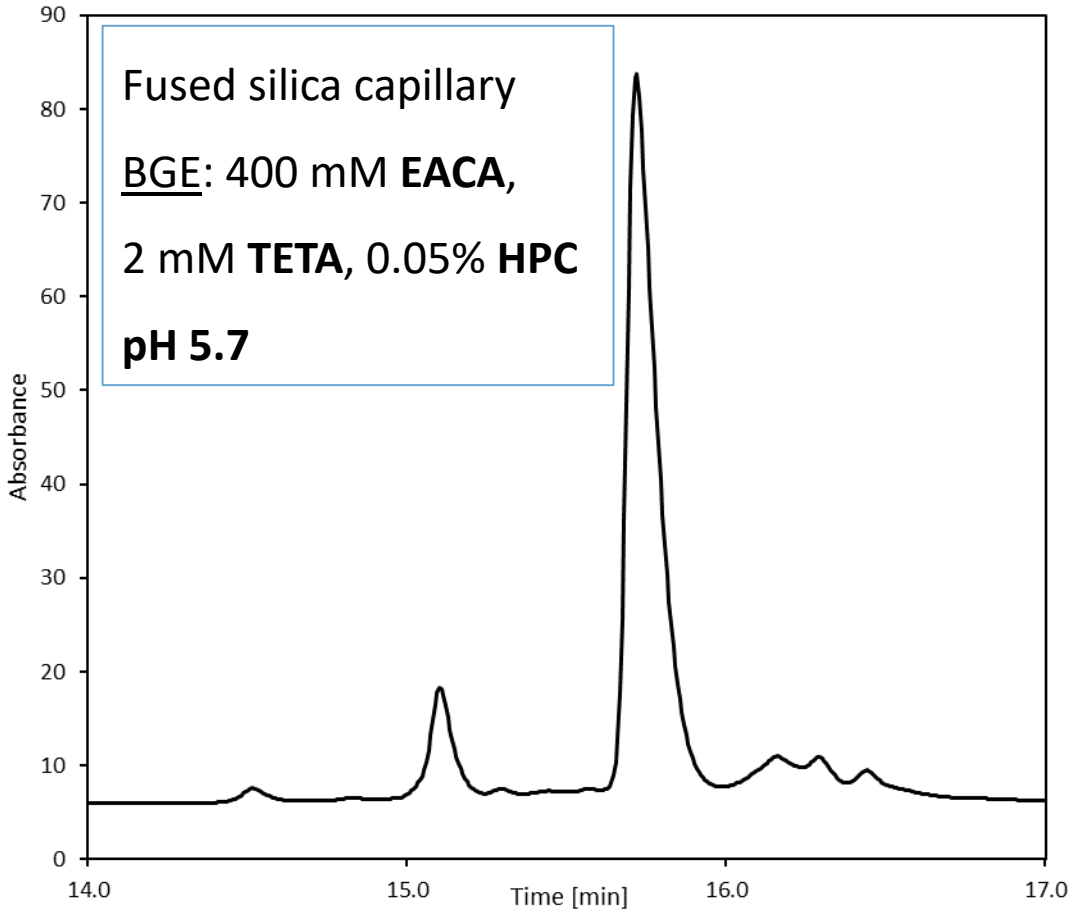
→ But: **Not** MS compatible ✗



→ MS compatible ✓

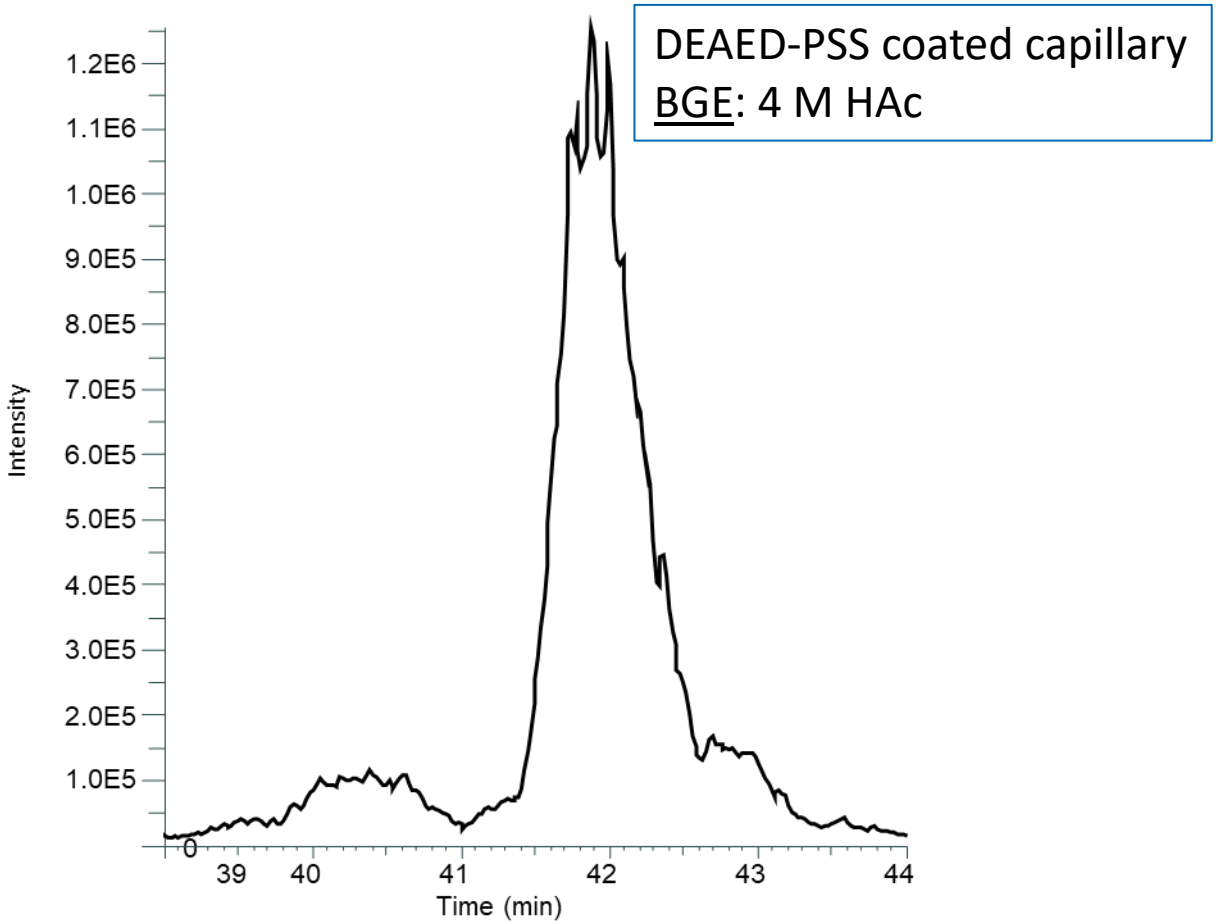
→ **No separation** of charge variants ✗

CE-UV of NISTmAb using different electrolytes



→ Efficient separation of charge variants ✓

→ But: **Not** MS compatible ✗

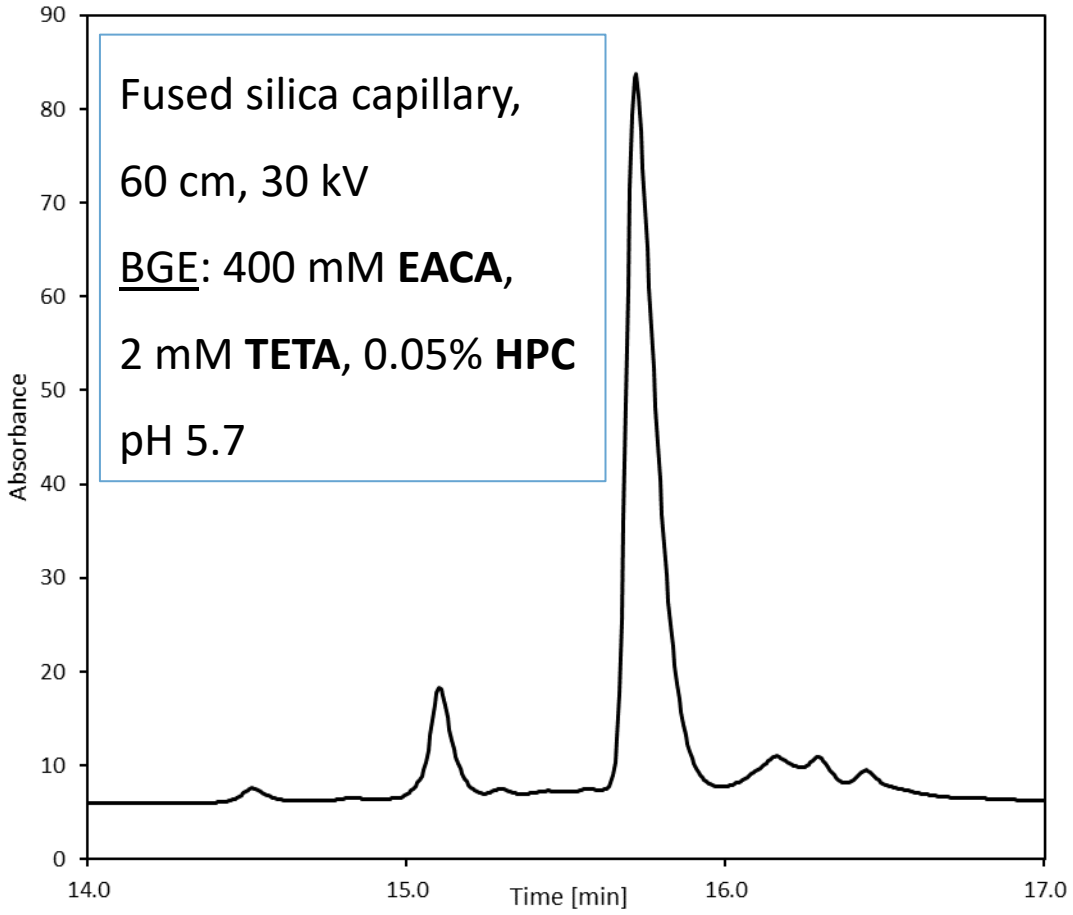


→ MS compatible ✓

→ **Slight separation** of charge variants

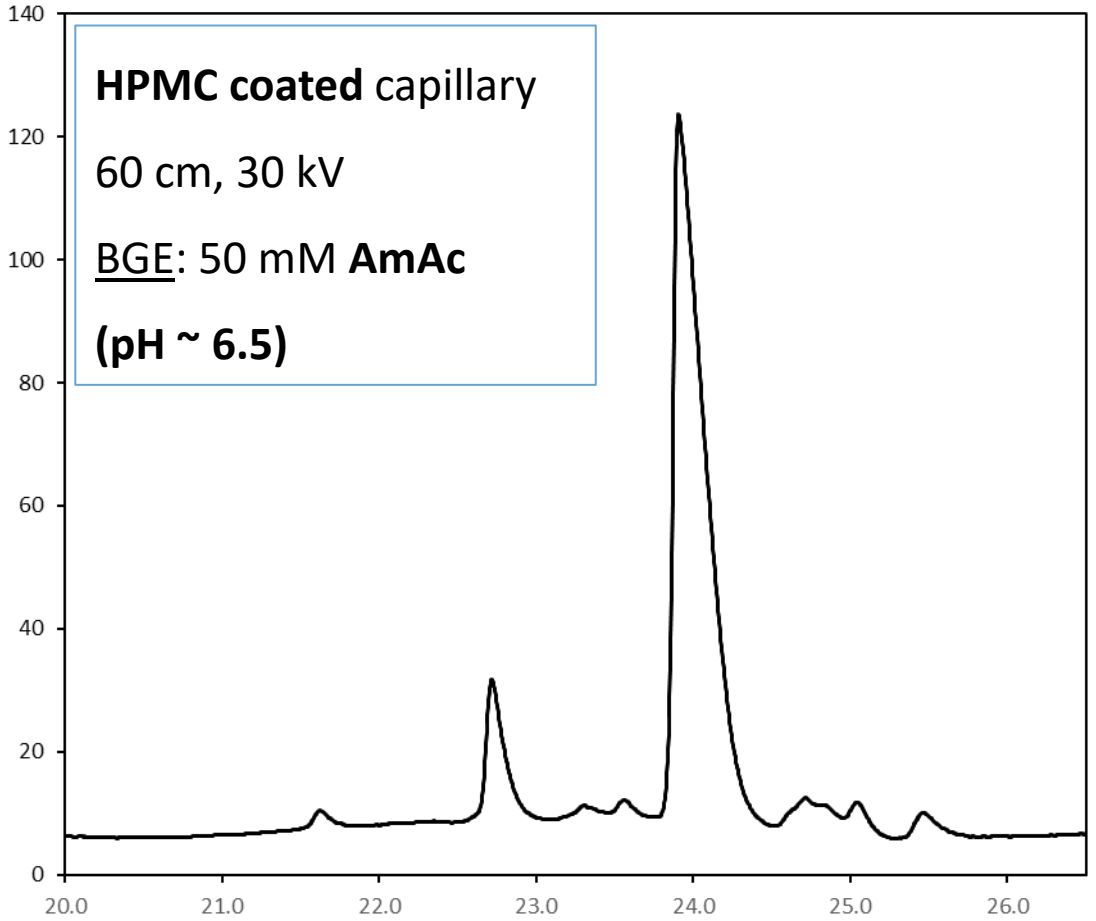
Höchsmann, A. et al., Charge variant analysis of monoclonal antibodies by CZE-MS using a successive multiple ionic-polymer layer coating based on diethylaminoethyl-dextran. *Electrophoresis* **2025**

CE-UV of NISTmAb using different electrolytes



→ Efficient separation of charge variants ✓

→ But: **Not** MS compatible ✗



→ Efficient separation of charge variants ✓

→ **MS compatible** ✓

CE-UV of NISTmAb using different electrolytes

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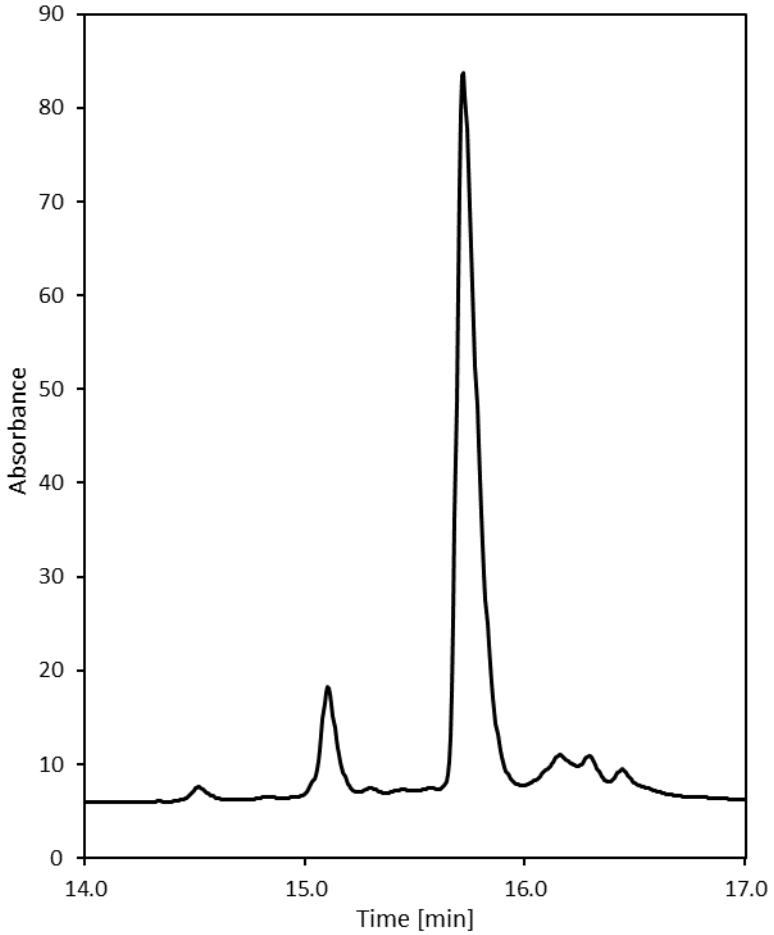
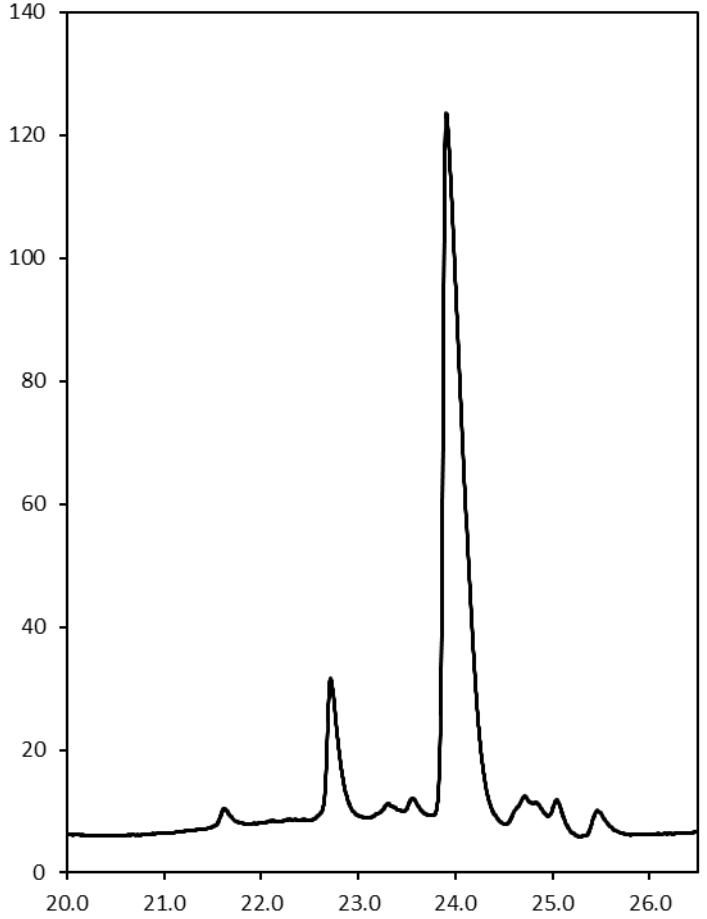
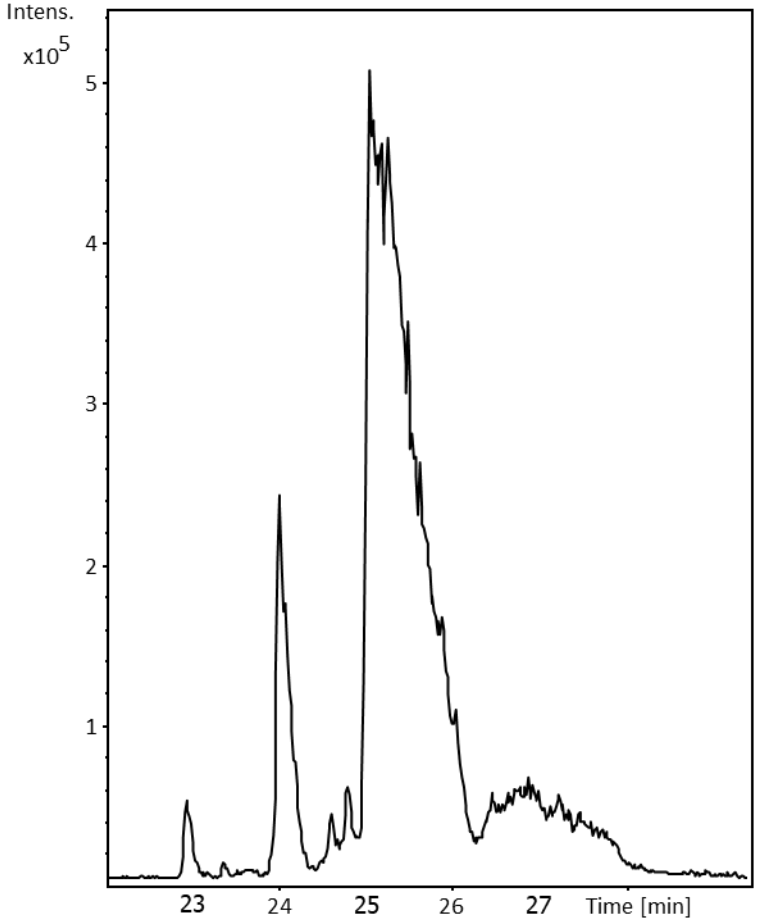
CE-UV (EACA/TETA method)



CE-UV (MS compatible method)



CE-MS (MS compatible method)



Fused silica capillary, 60 cm, 30 kV

BGE: 400 mM **EACA**, 2 mM **TETA**, 0.05% **HPC;** pH 5.7

HPMC coated capillary, 60 cm, 30 kV

BGE: 50 mM **AmAc (pH ~ 6.5)**

Repeatability and Reproducibility of HPMC coated capillaries

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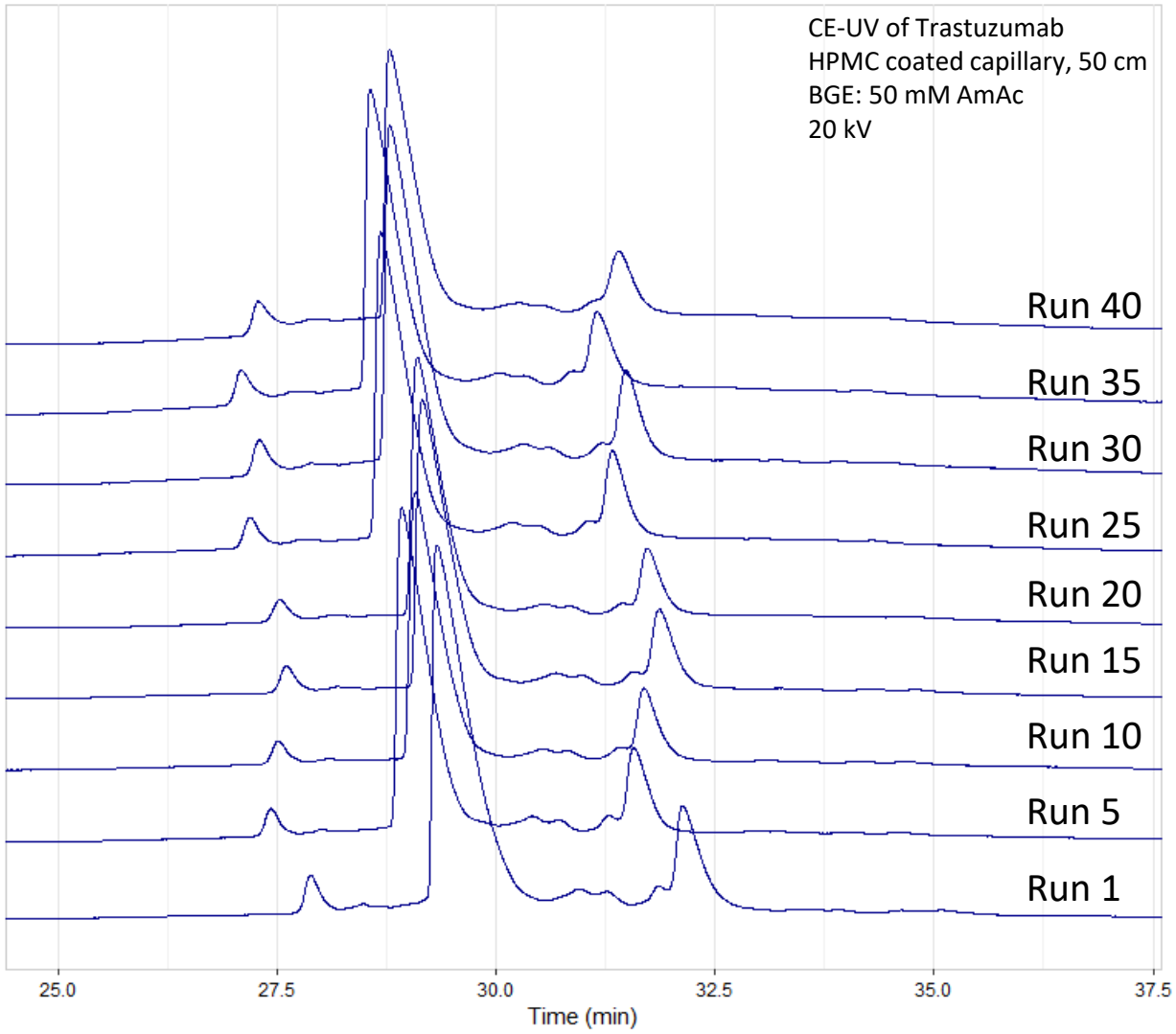
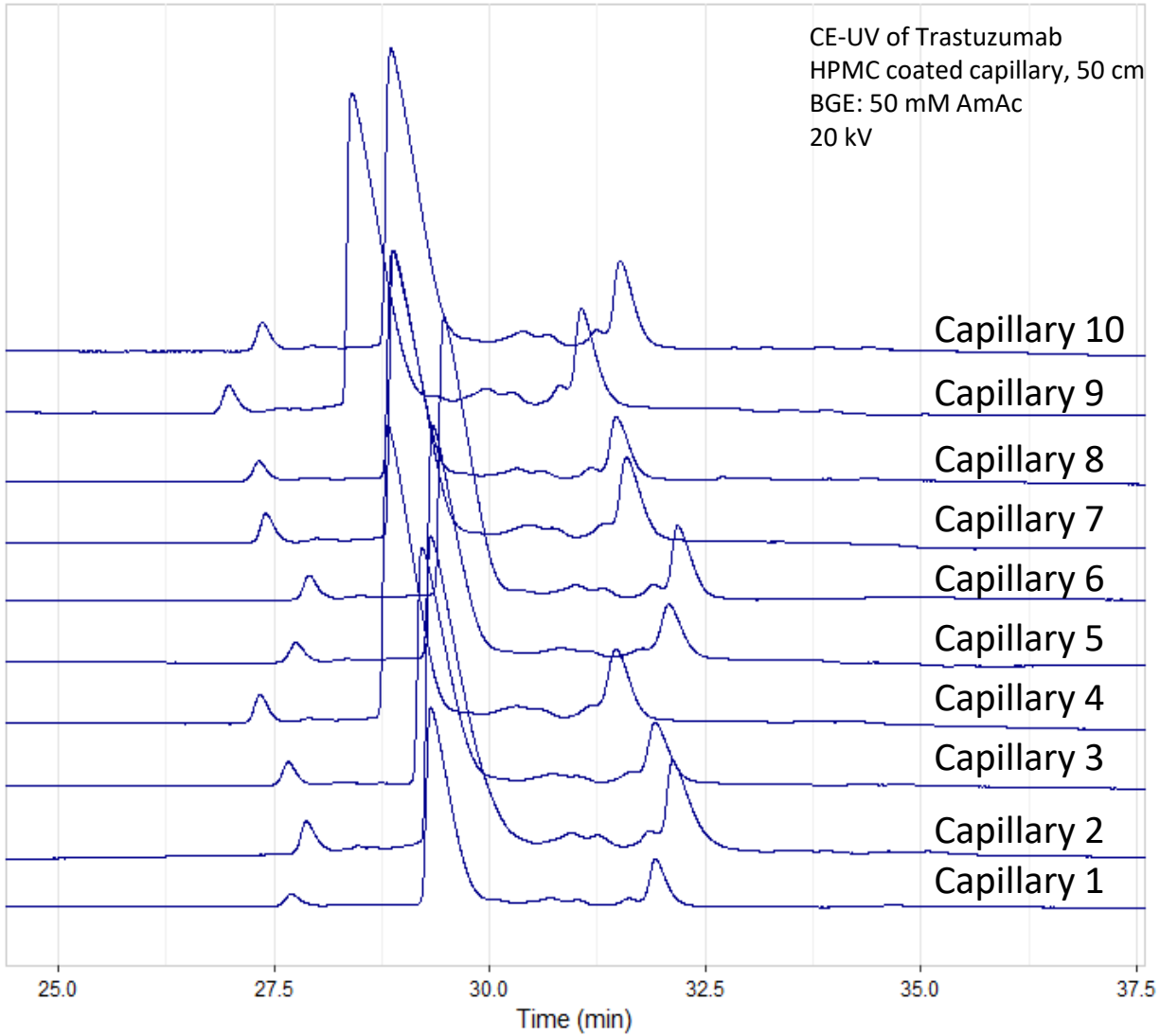
Repeatability (40 runs)



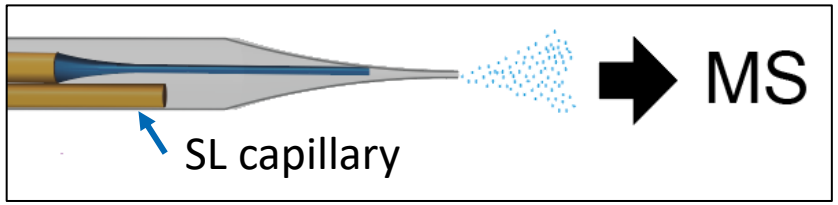
Relative standard deviation of migration times: 0.96%

Reproducibility (10 capillaries)



Relative standard deviation of migration times: 1.15%

CE-MS using the nanoCEasy interface



→ different SL compositions can be applied

Sheath liquid capillary

HPMC coated capillary

MS

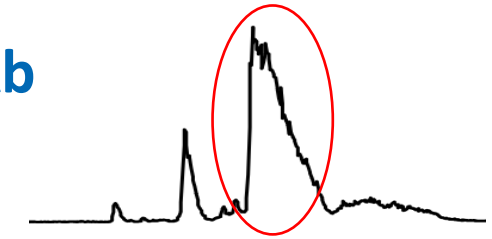
CE voltage

ESI voltage

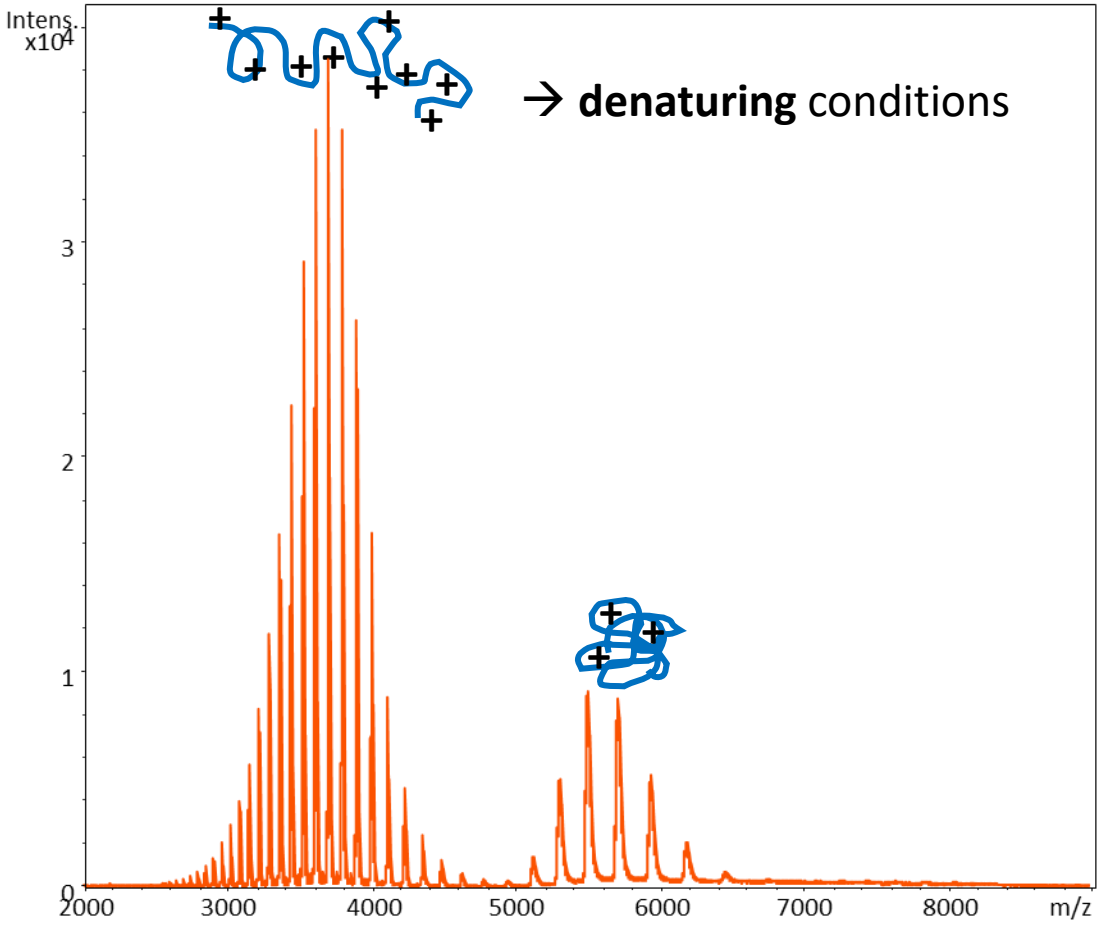
Electrolyte: 50 mM AmAc

J. Schlecht et al., nanoCEasy: An Easy, Flexible, and Robust Nanoflow Sheath Liquid Capillary Electrophoresis-Mass Spectrometry Interface Based on 3D Printed Parts, *Anal. Chem* **2021**

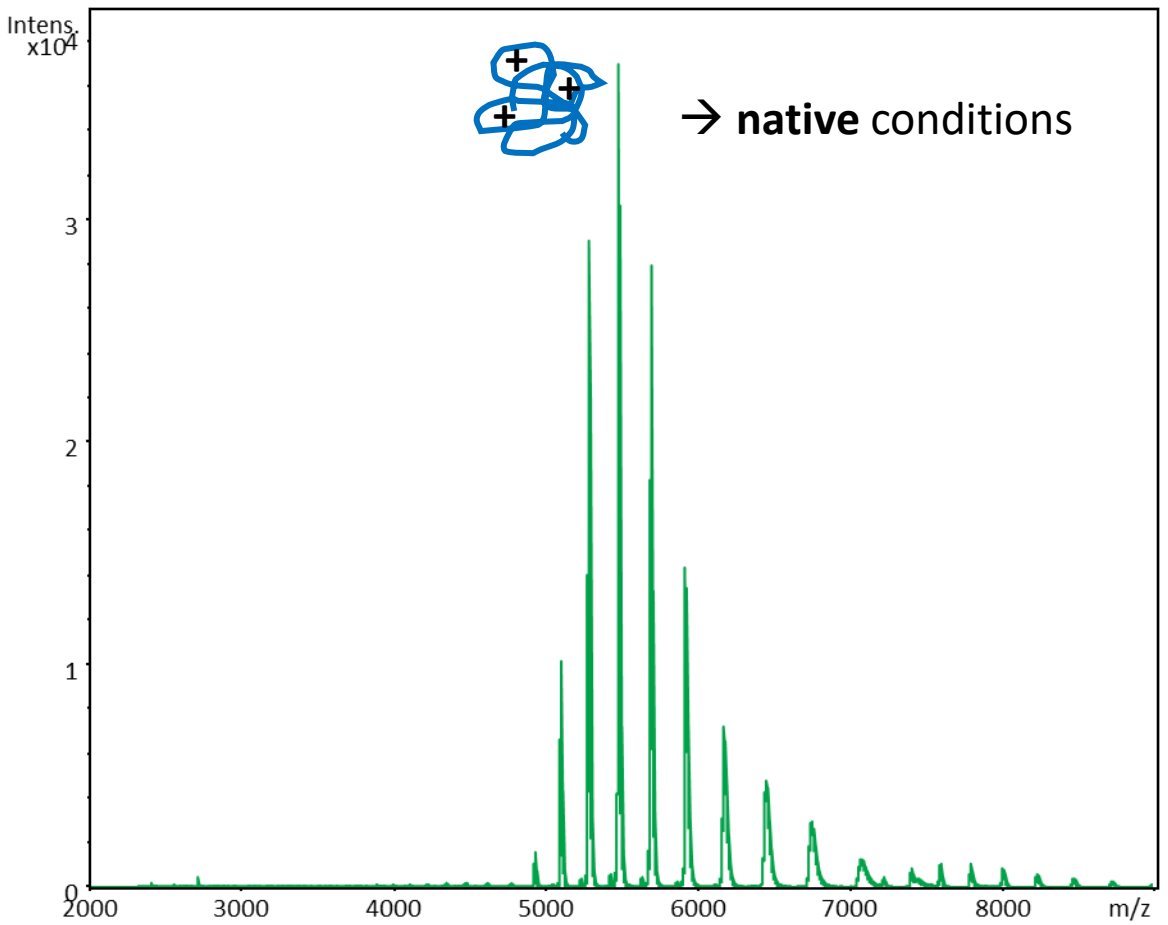
CE-MS of the main form of NISTmAb using different SL



IPA/H2O (50:50), **5 % HAc**

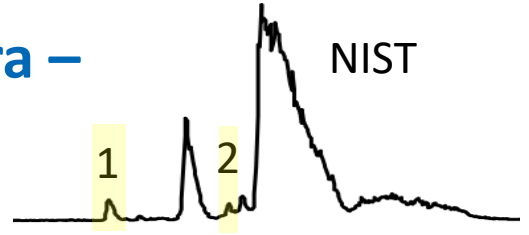


IPA/H2O (**30:70**), **20 mM AmAc**



→ The nanoCEasy interface enables analysis of CE separated charge variants under **denaturing** and under **native nanoESI conditions**

Comparison deconvoluted mass spectra – denaturing vs. native ESI conditions



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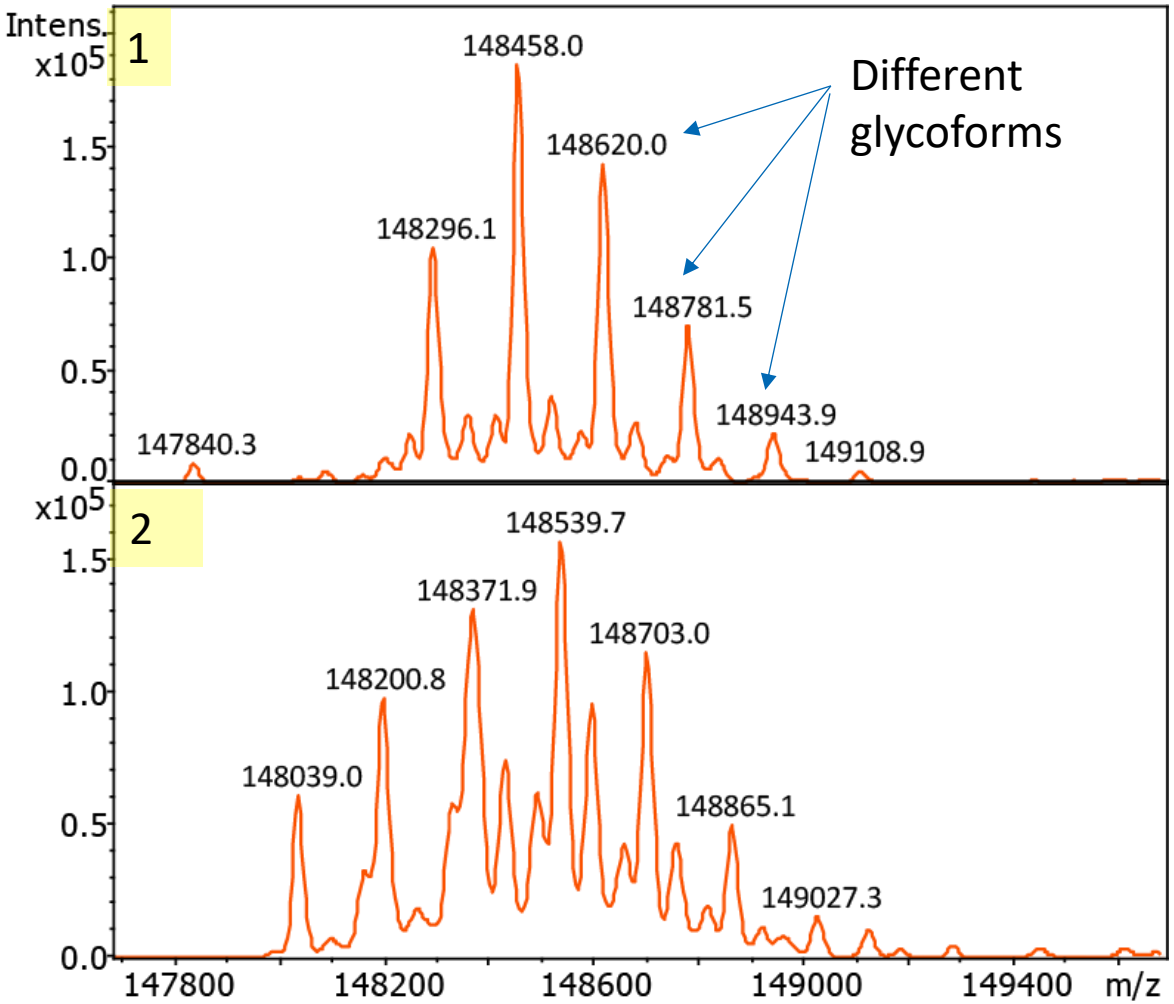
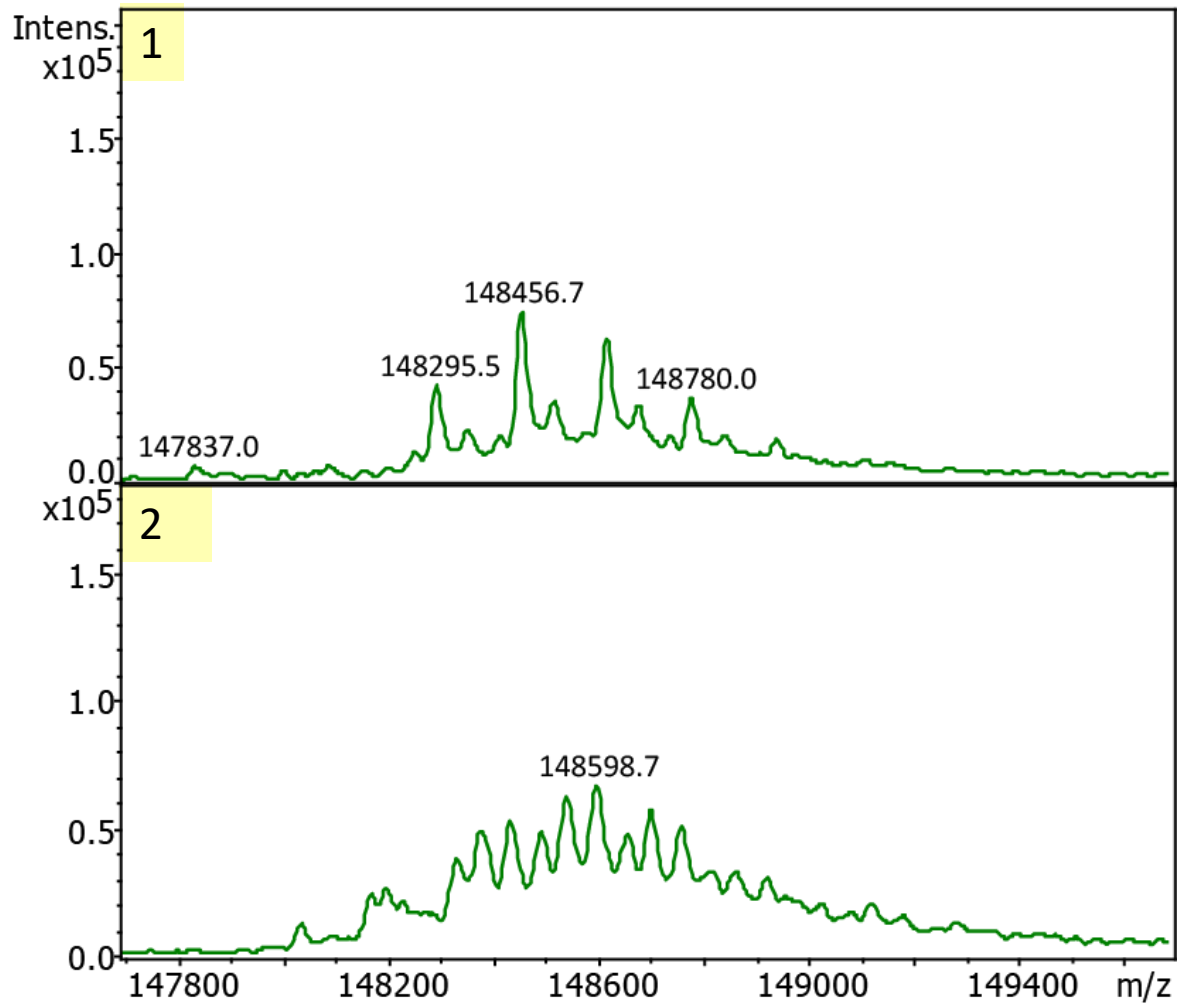
Denaturing

SL: : IPA/H2O (50:50), 5% HAc



Native

SL: : IPA/H2O (30:70), 20 mM AmAc



CE-MS of different mAbs under native separation conditions

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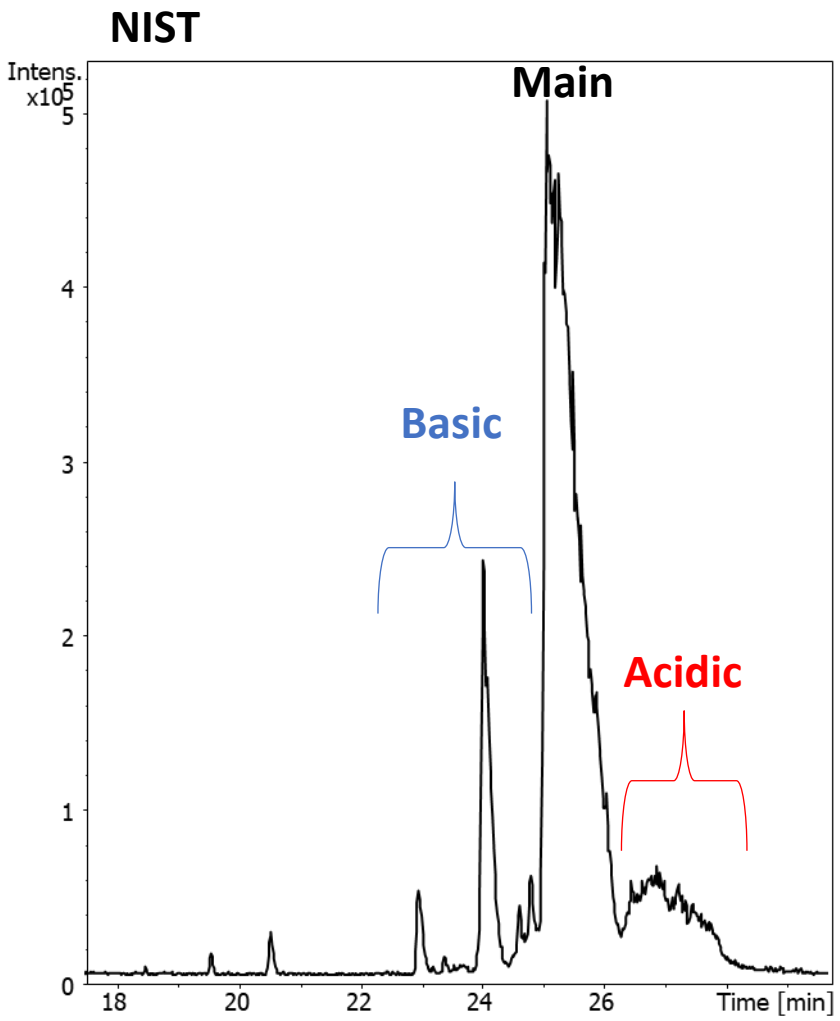
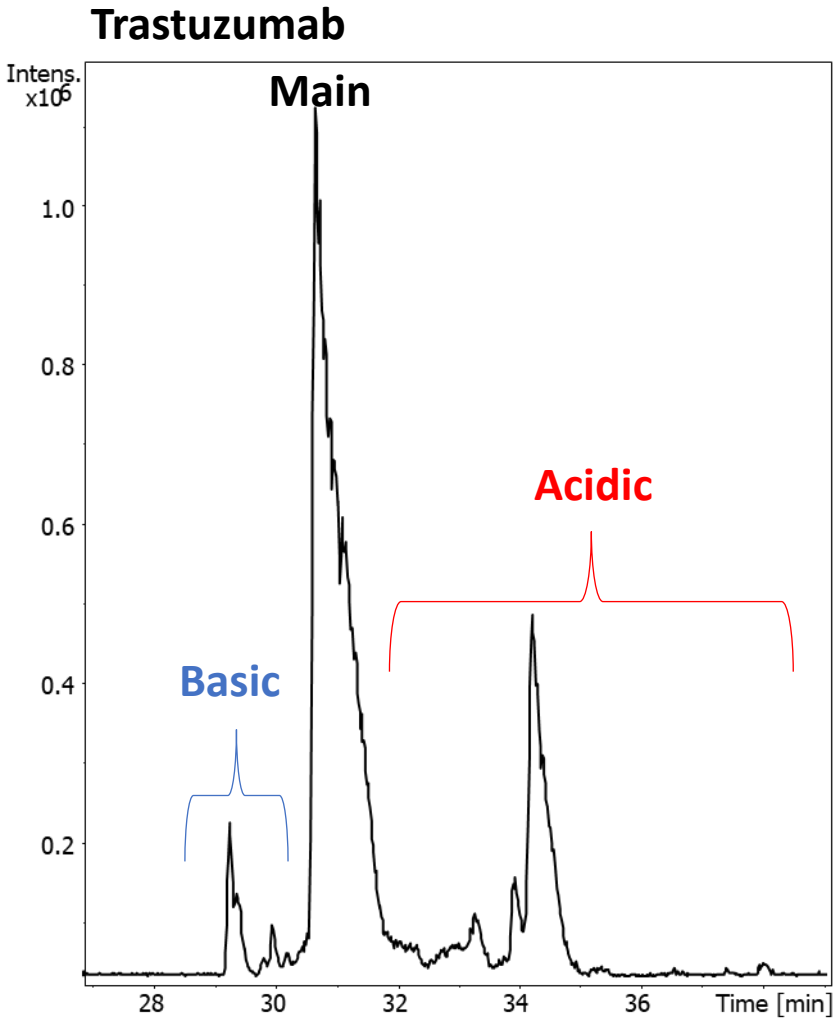
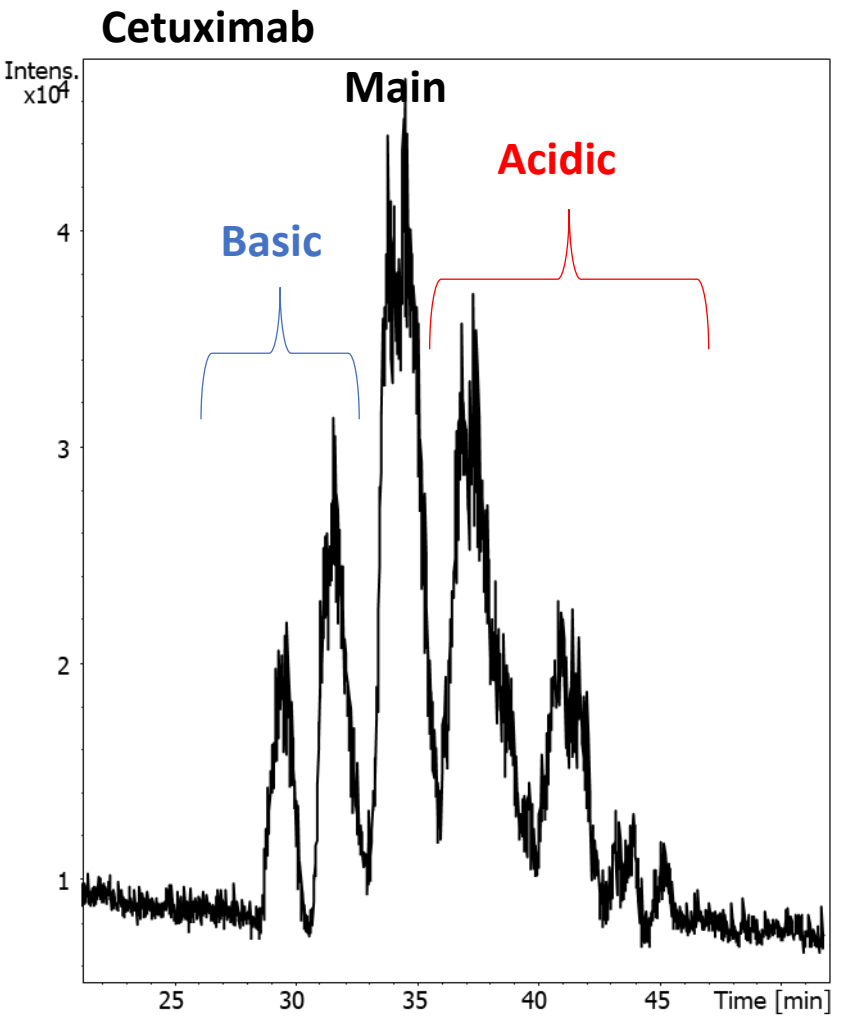
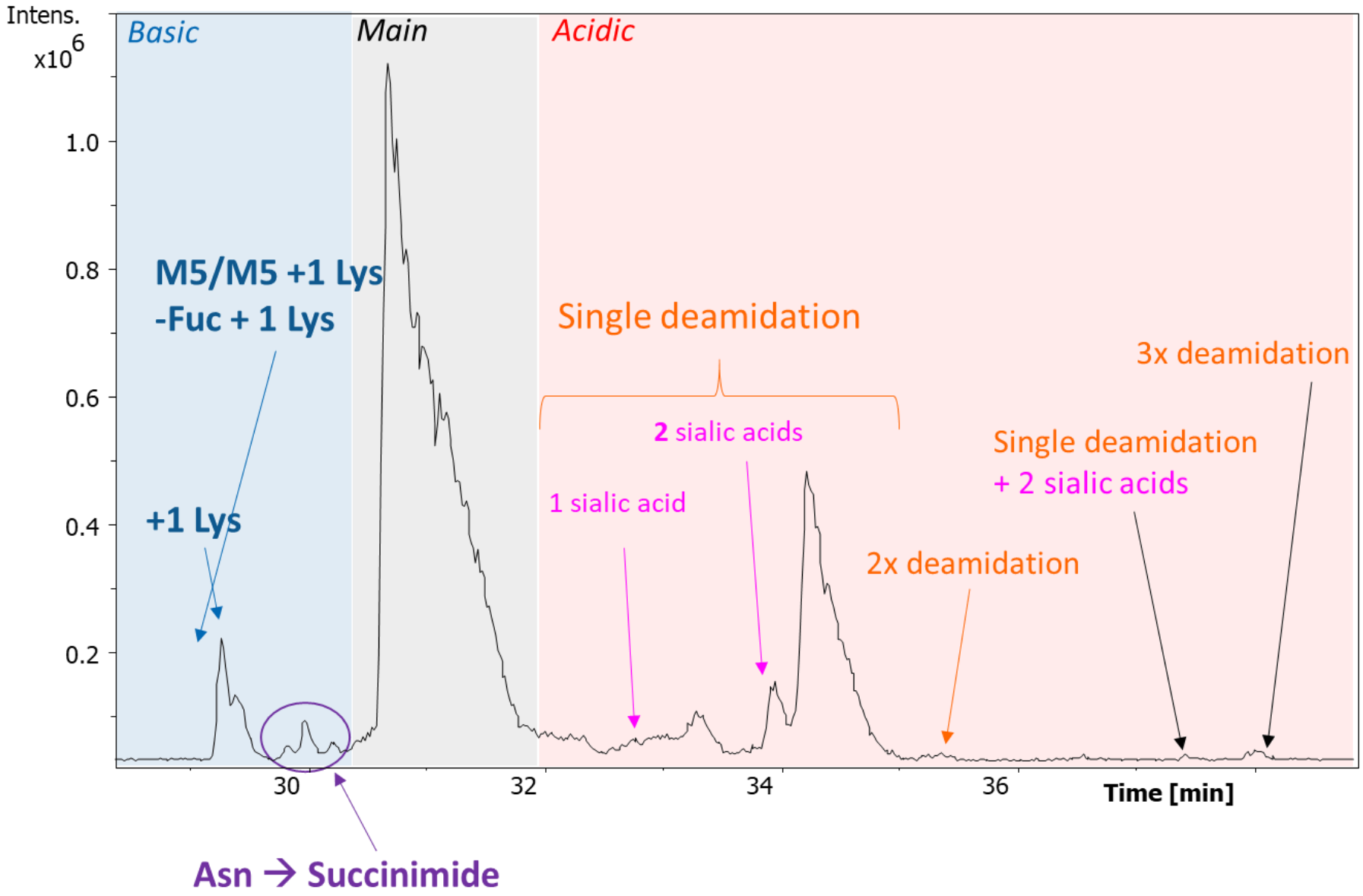






→ separation of basic, main and acidic variants

Characterization of Trastuzumab by CE-MS



Characterization of Trastuzumab and NISTmAb by CE-MS

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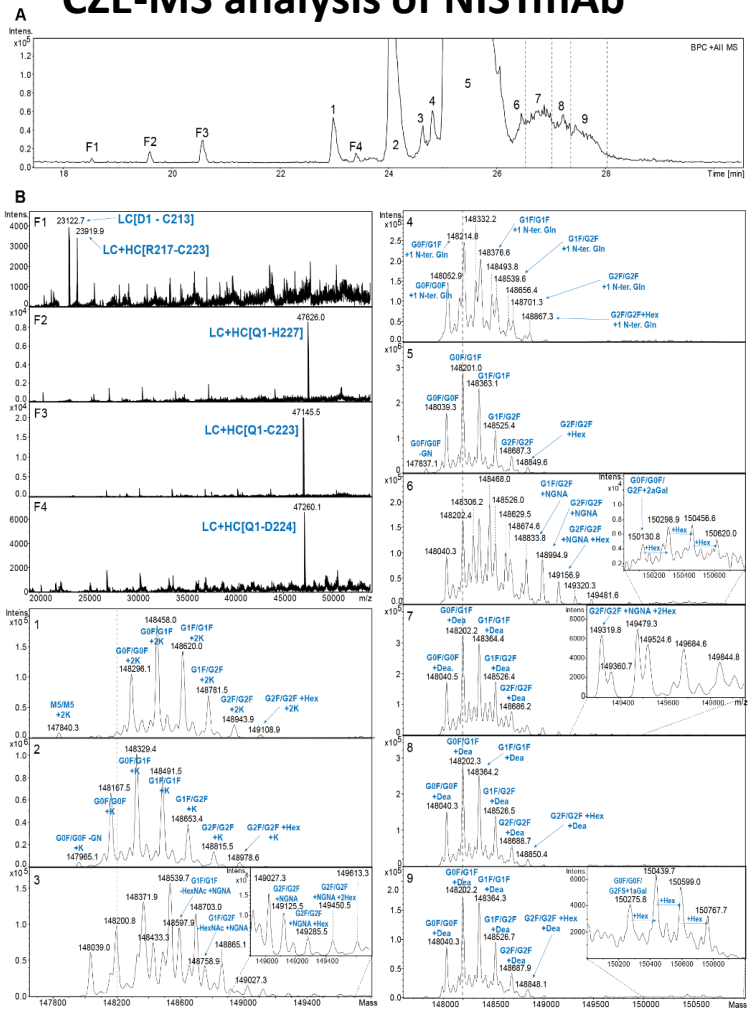
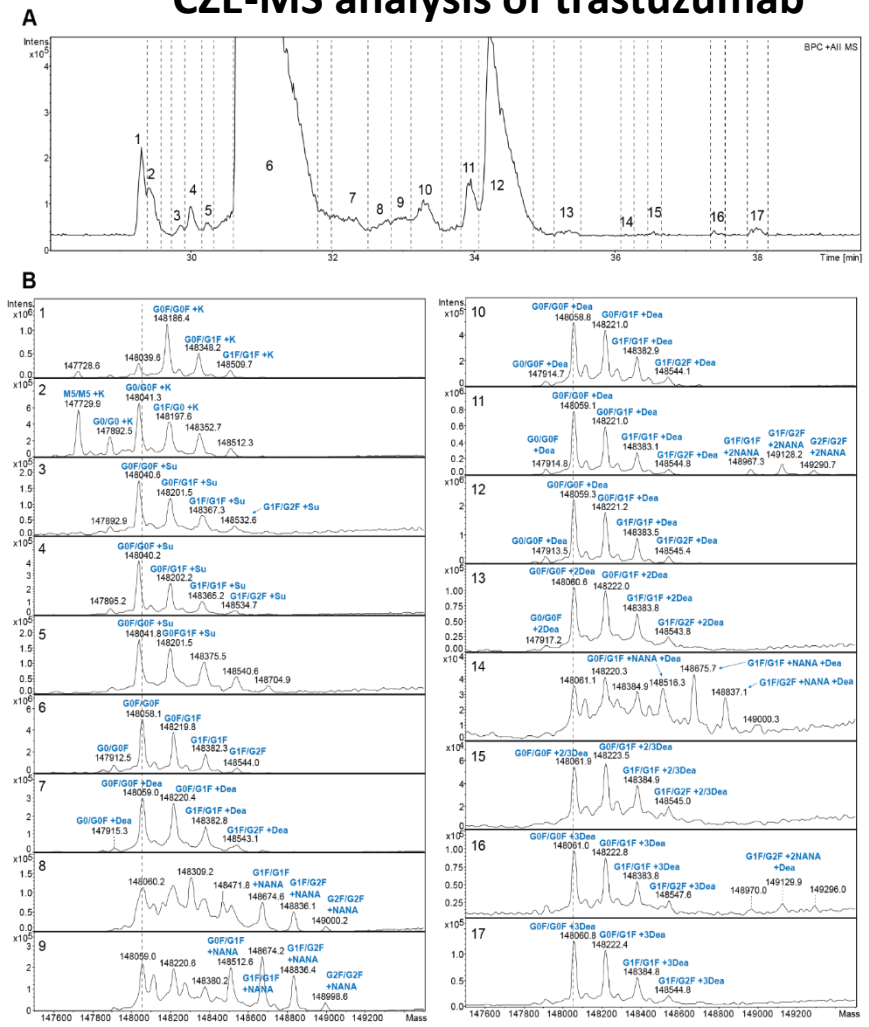
CZE-MS analysis of NISTmAb



CZE-MS analysis of trastuzumab



→ Detection of **70 proteoforms** for **trastuzumab** and **115 proteoforms** for **NISTmAb**

Schwenzer, A. K., & Neusüß, C., High-Performance Native Separation of mAb Proteoforms by CZE-MS under Native and Denaturing nanoESI Conditions for Ultrasensitive Charge Variant Characterization. *Anal. Chem* **2025**

Ion Mobility of native mAbs on modified timsTOF SCP

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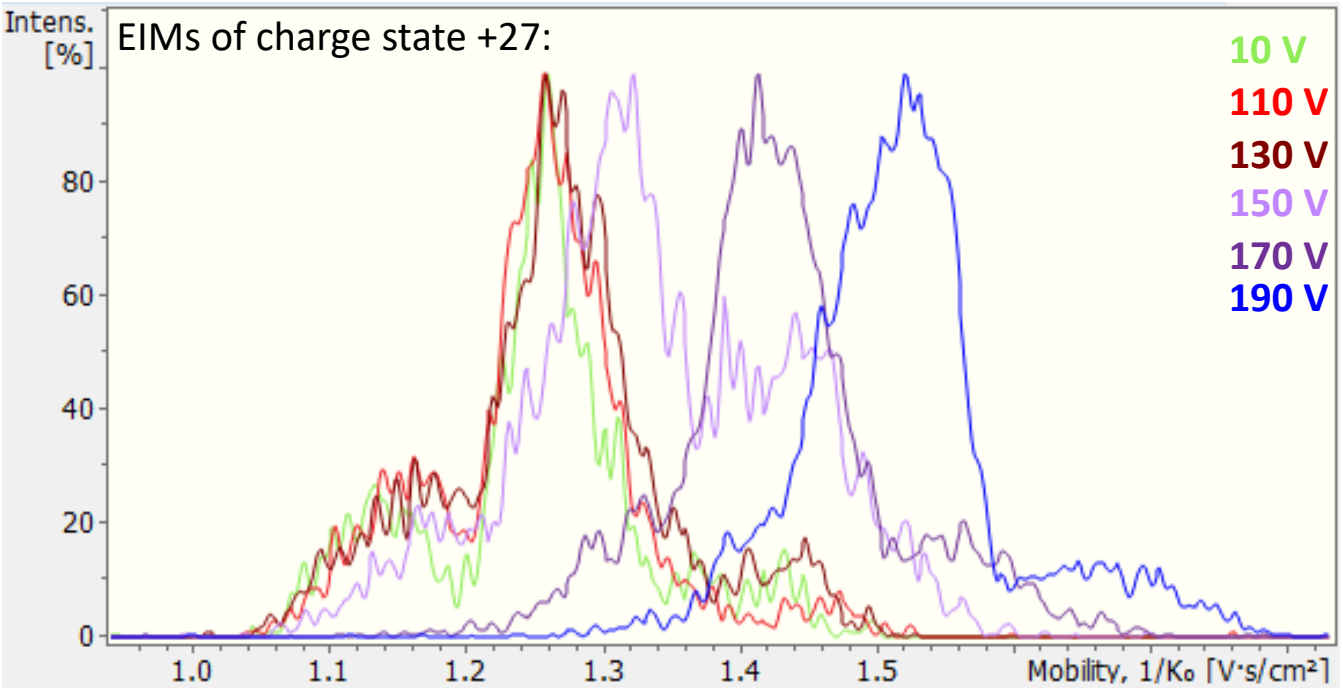
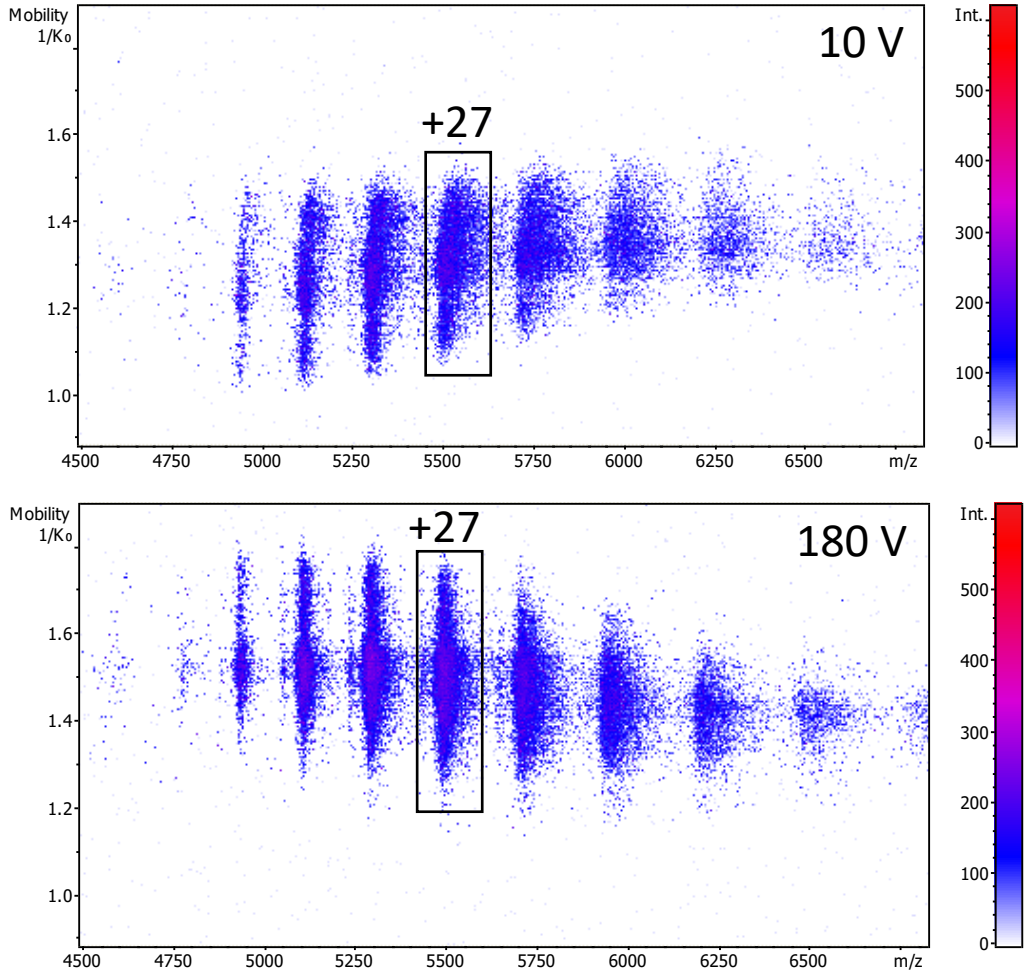
→ Collision Induced unfolding (CIU) of Trastuzumab:





Changes in structure of protein variants?

→ Differences in mobility

→ Differences in CIU

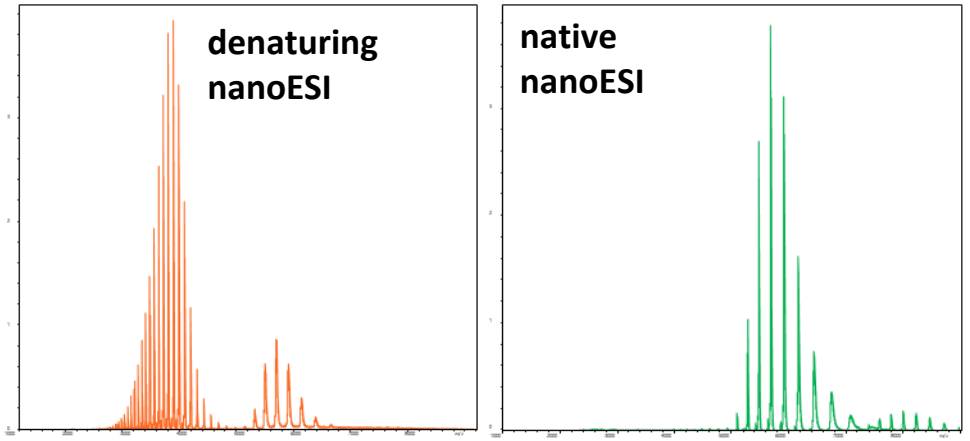
Conclusion & Outlook

- Combination of native CE separation with ultrasensitve denaturing online MS analysis

  for charge heterogeneity characterization

→ Detection of **70 proteoforms** for trastuzumab and **115 proteoforms** for **NISTmAb**

- **Online MS analysis under denaturing or native ESI conditions**



<u>Outlook:</u>

- Native SL system enables **affinity CE with native MS**

→ Analysis of **antibody-antigen interactions** of **proteoforms**?

- Analysis of **changes in structure of protein variants** using ion-mobility MS?

Acknowledgement

Founding: German Federal Ministery for Education and Research (FKZ 13FH135KX0)

Bundesministerium
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und Forschung

FORSCHUNG AN
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Collaboration Partners:

abbvie

Martin Eisinger

Nikolai Hentze

Markus Hollmann

BRUKER

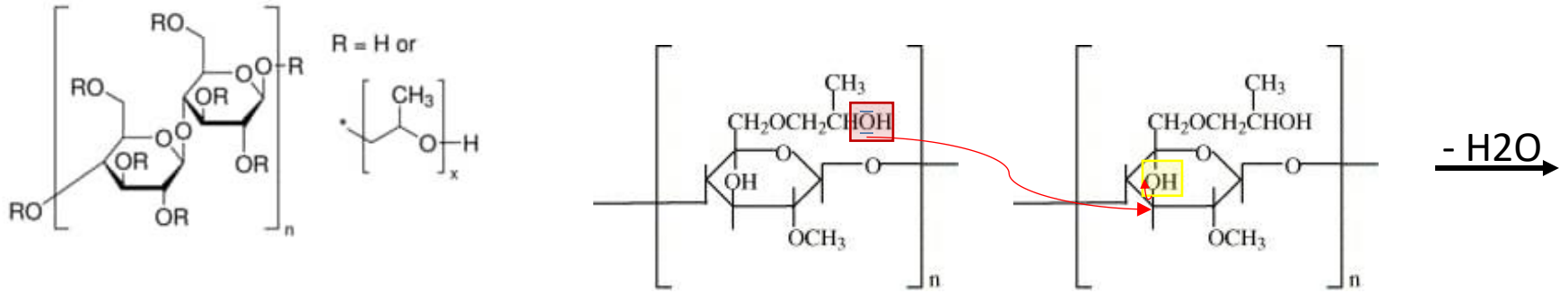
Stuart Pengally

Oliver Räther

Christian Albers

Immobilization of HPMC in the capillary

→ By heating, the **hydroxyl groups on the substituted hydroxyalkyl chains** can **react** with the **remaining hydroxyl groups** on the substituted cellulose chain to yield relatively pH stable cellulose ether bonds, as formed in the manufacture of the hydroxyalkyl-substituted celluloses. Shen et al. **suppose** that chemical **reactions** occur **between** the coated hydroxyalkyl-substituted **celluloses** and the **fused silica capillary** inner wall.



Comparison of CE currents for different SL:

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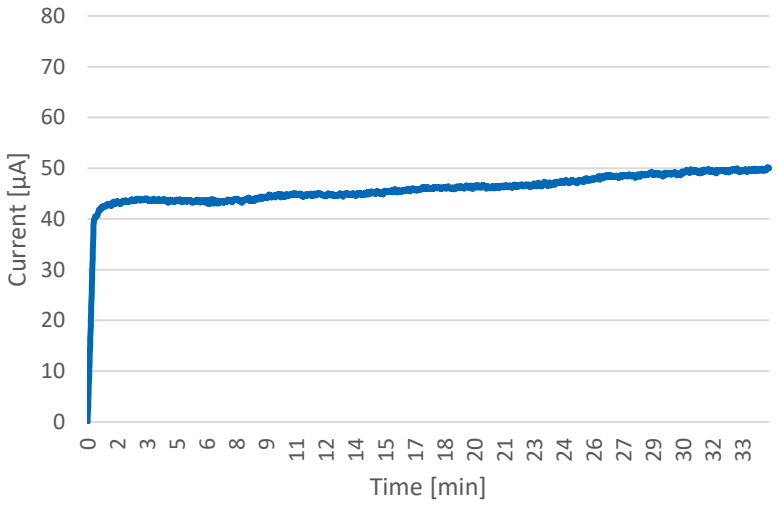
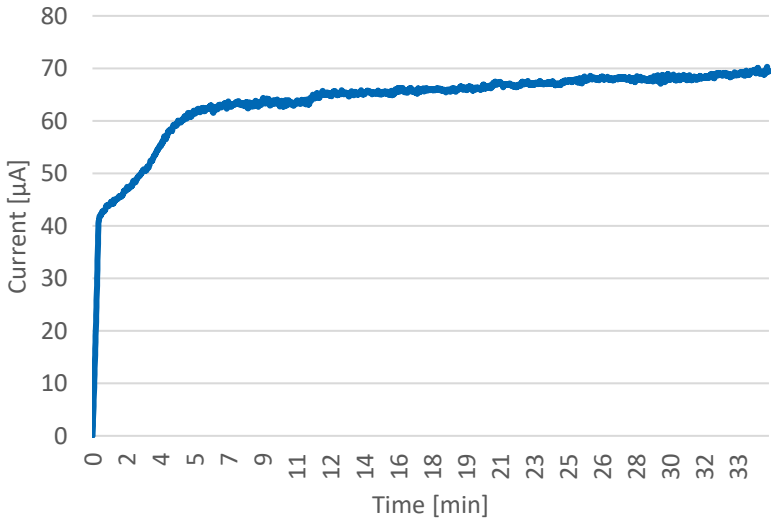
Current for SL
IPA/H2O(30:70), 20 mM AmAc



Current for SL
IPA/H2O(1:1), 0.5% FA



Current for SL
IPA/H2O(1:1), 5% HAc