Coupling imaged Capillary Iso-Electric Focusing with Mass Spectrometry (*i*CIEF-MS)

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Iso-Electric Focusing Applications

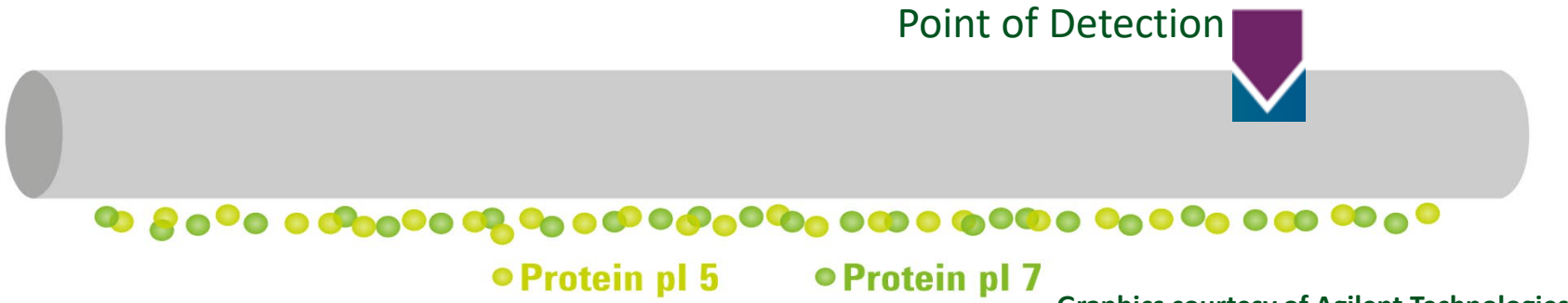
- → Versatile, essential separation method for the analysis of proteins
- → In proteomics for:
  - Determination of the pI of an unknown peptide or protein
  - Assessment of charge heterogeneity, purity or presence of isoforms of proteins
  - Determination of PTMs (de-amidation, oxidation) of recombinant proteins, mABs and antibody drug conjugates (ADC)
- → In manufacturing of recombinant proteins for:
  - Product purity and identity
  - Product stability
- → In clinical analysis for:
  - Analysis of serum proteins
  - Determination of hemoglobin variants
  - Glycosylation of transferrin
- → In food analysis for:
  - Identify and characterize allergenic proteins

Iso-Electric Focusing

- → Planar Format
  - Slab gel
  - Immobilized pH gradient (IPG, Righetti et al.)
  - IEF strips for 2D GE
- → Capillary Format

IEF → separation in

Principles of Capillary IEF



At the beginning of a CIEF run, the whole capillary (PFE coated fused silica) is filled with a mixture containing:

- Carrier Ampholytes (CA, polyamino-polycarboxylic acid oligomers with pI's ranging from 3-10 and MW 500-800)*
- Protein or peptide sample to be analyzed (amphoteric molecules)
- pI markers (amphoteric molecules)
- All molecules will be homogeneously distributed over the whole capillary at start. pH of the solution average of all pKa's and pKb's; molecules with a **low pI** will have a positive charge, molecules with **high pI** have a negative charge at start
- The inlet side low pH (anolyte, H_3PO_4), high positive voltage, anode
- The outlet side high pH (catholyte, NaOH), grounded, cathode
- Apply voltage/field

*P. Righetti, Isoelectric Focusing: Theory, Methodology and Application, 1st Edition, 1983, Elsevier Science Publishers

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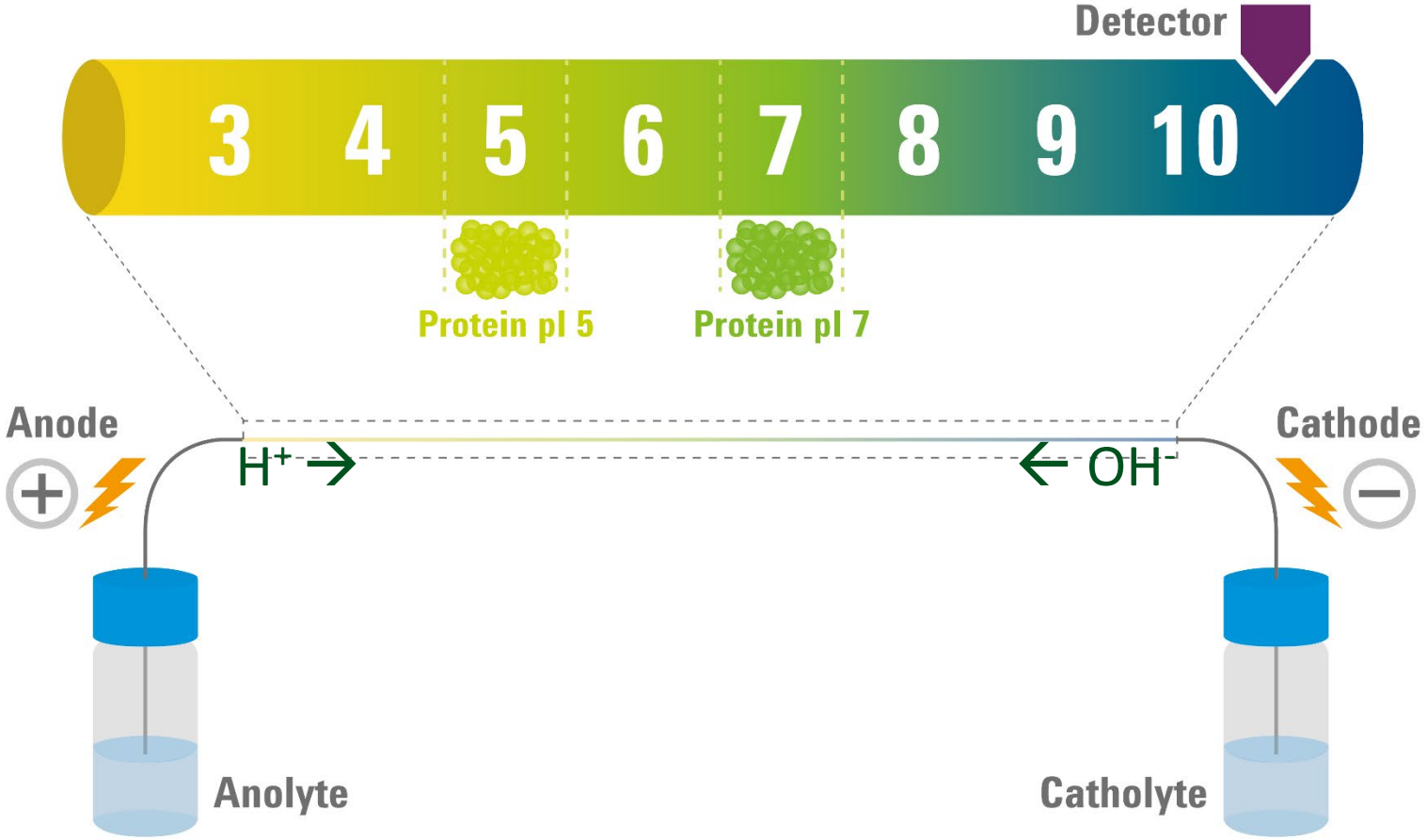
CIEF, Focusing in Front of the Point of Detection!

Sacrificing ampholytes are needed to keep the focused zones within the capillary and before PoD!!!
Focused protein zones need be moved over the PoD to become quantified!!



Graphics courtesy of Agilent Technologies

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Capillary Iso-Electric Focusing

Advantages:

- Uses existing commercial CE instruments
- Delivers quantitative data/electronic records

Challenges:

- Keep protein zones in front of the PoD during focusing (Sacrificing ampholytes)
- Minimize resolution loss during mobilization

Drawbacks:

- Two step process → long analysis time (0.5-1 hour/run)
- Method development long and complicated

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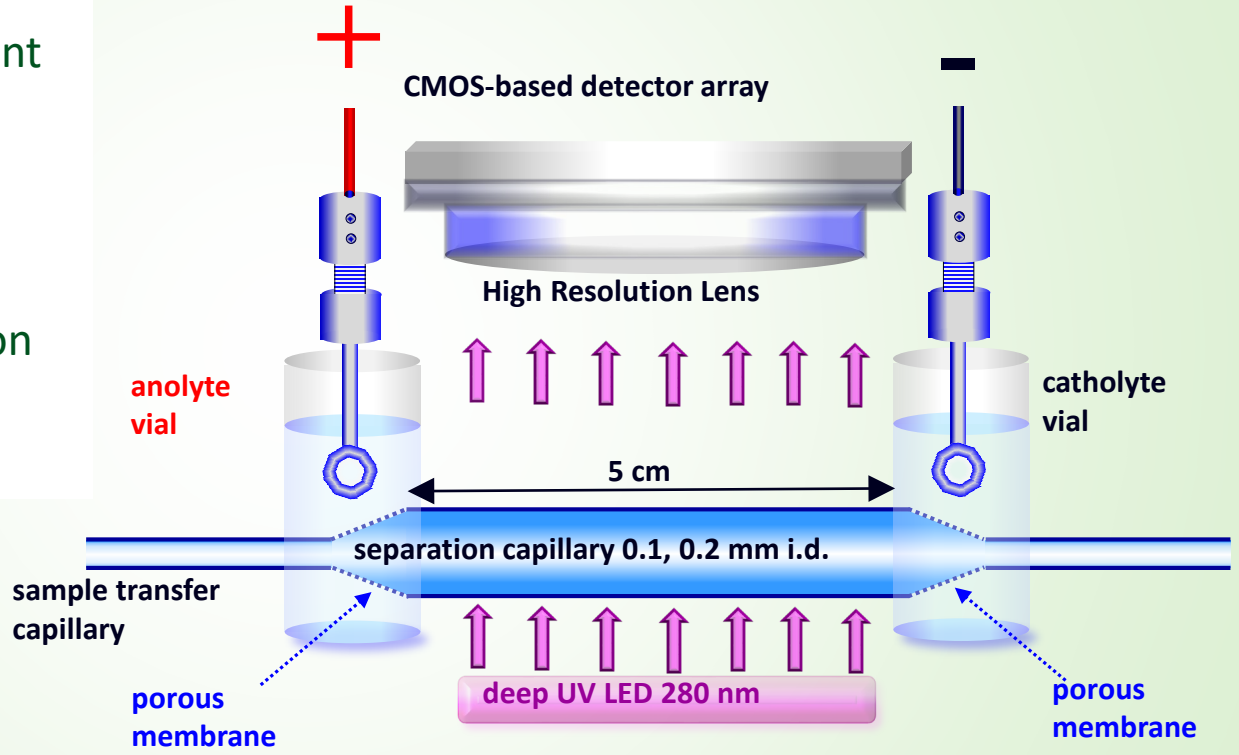
ISC 2018 Cannes-Mandelieu, France
32nd International Symposium on Chromatography
September 23-27, 2018

CEInfinite for *imaged* CIEF by AES Ltd.

- First described by Pawliszyn and Wu, Anal. Chem., 1992, **64**, 224-227.
- Commercialized by Convergent Bioscience in 2000, iCE280.
- Convergent acquired by Cell Biosciences (now Protein Simple) in 2010.
- New market *i*CIEF introduction by Advanced Electrophoresis Solutions in 2016



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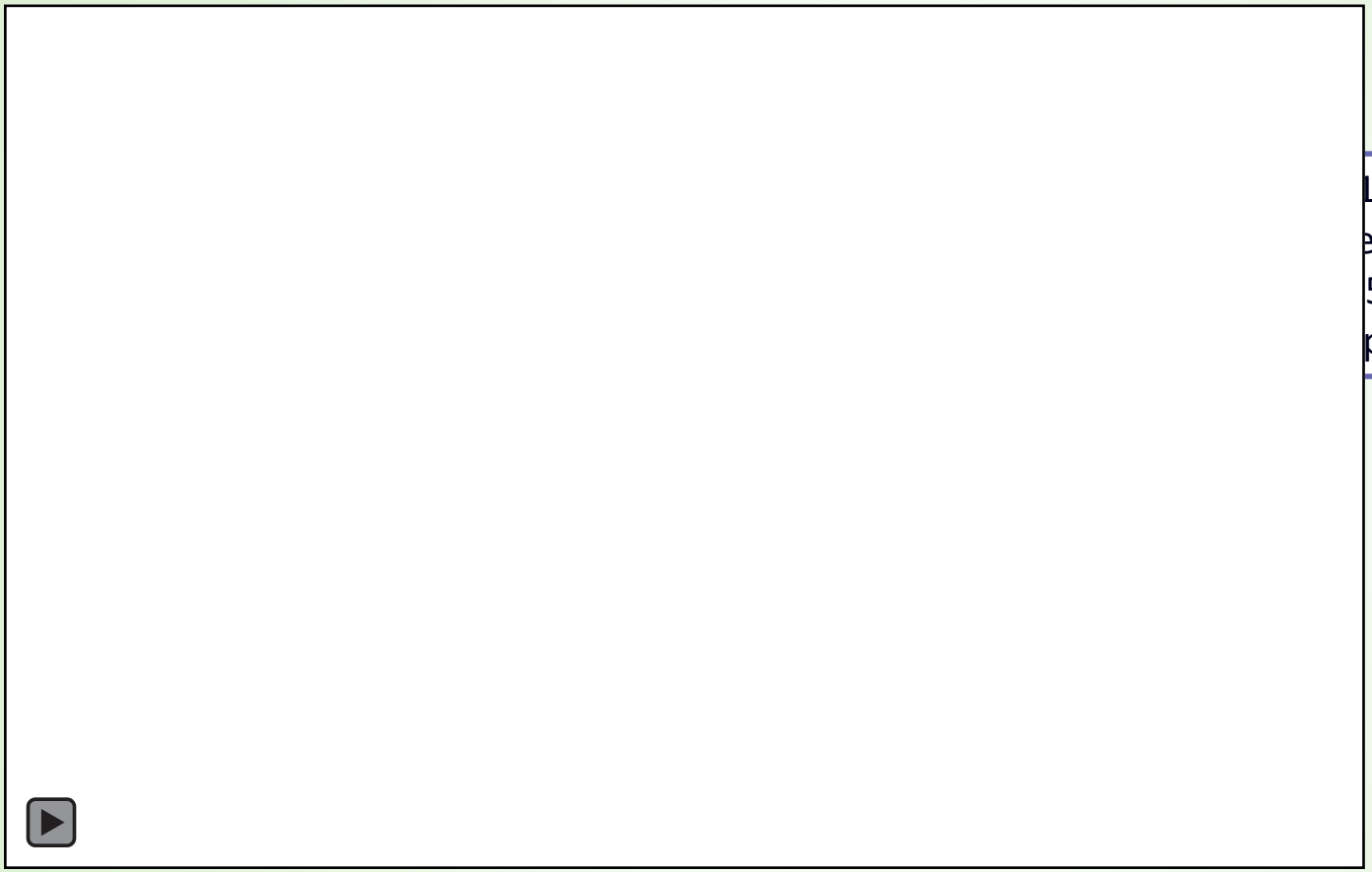
Separation of Hemoglobin Isoforms



10
e Hemoglobin Standard
5, Focus. Time 3’
pillary 50x0.1 mm

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Challenges in Coupling *i*CIEF with ESI-MS*

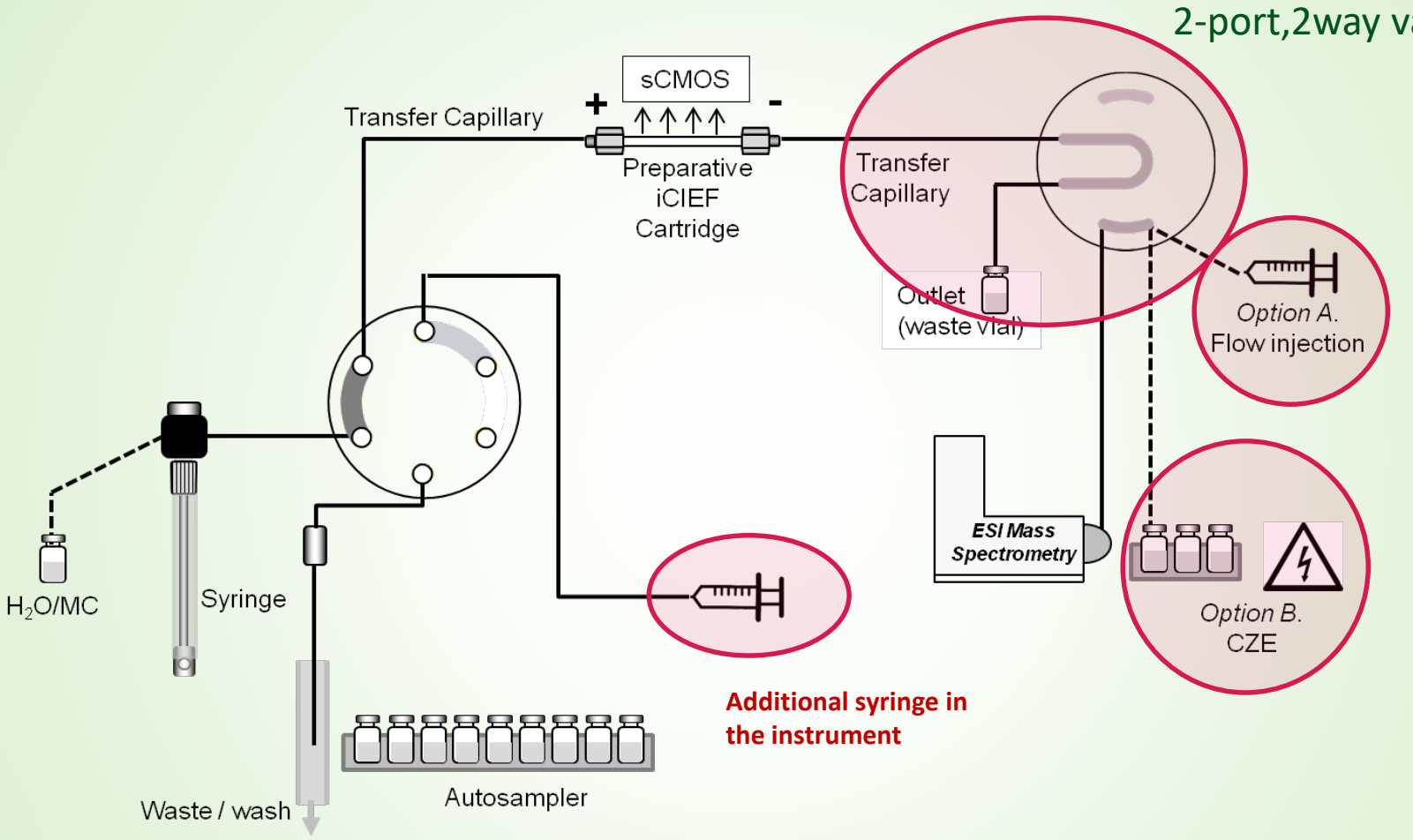
- → Transport separated, nanoliter size zones to the ESI interface
  - Chemical mobilization ??
  - Extra syringe for pressure mobilization maintaining focusing voltage
- → Interference/ion suppression of the ESI process by ampholytes
  - Localized separation - proteins and CA have different pI and are in different positions
- → Non-volatile neutral additives (methyl cellulose) will cause contamination of the MS inlet
  - Polyacrylamide coated separation capillary does not require to add MC
- → Deal with two high voltage sources on one liquid conductor
  - CE-MS IF from Bruker and Agilent MS have sprayer needle at ground
- → Focused zone has up to 50x higher protein concentration
  - Keep protein in solution
  - Avoid dispersion during transport → much lower i.d. transfer capillary

***Dai et al., Anal. Chem. 2018, 90, 2246−2254**

Approaches for iCIEF-MS Coupling

- ✓ Coupling with sampling valve – Heart-cutting approach
  - ➢ Neusüß et al.
- ✓ Direct coupling with ESI using sheath solvent
  - ➢ Huang et al.

*i*CIEF-MS with Nanoliter Samping Valve*



*C. Montealegre & C. Neusüß, Electrophoresis 2018, 39, 1151–1154

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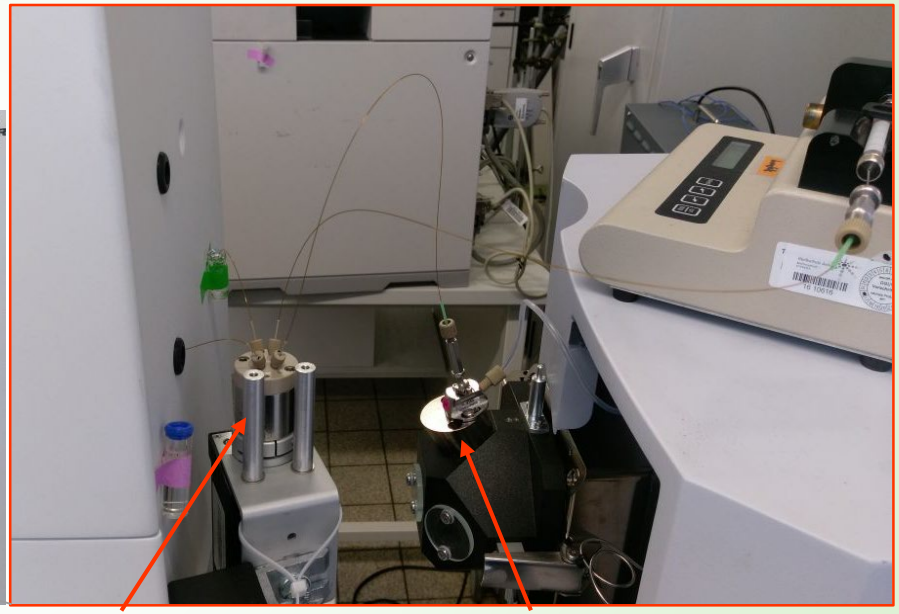
Coupling iCIEF with MS by the 4-port Nanoliter Valve

1st D: iCIEF, CEInfinite

Flow injection-MS



Valve

microLC sprayer

- Easy hydraulic coupling of CEInfinite with nanoliter valve, and Bruker MS
- In this configuration CE and ES voltages are decoupled

  ➔ no dedicated CE-MS interface required, µHPLC or nanoHPLC ESI interface suffices

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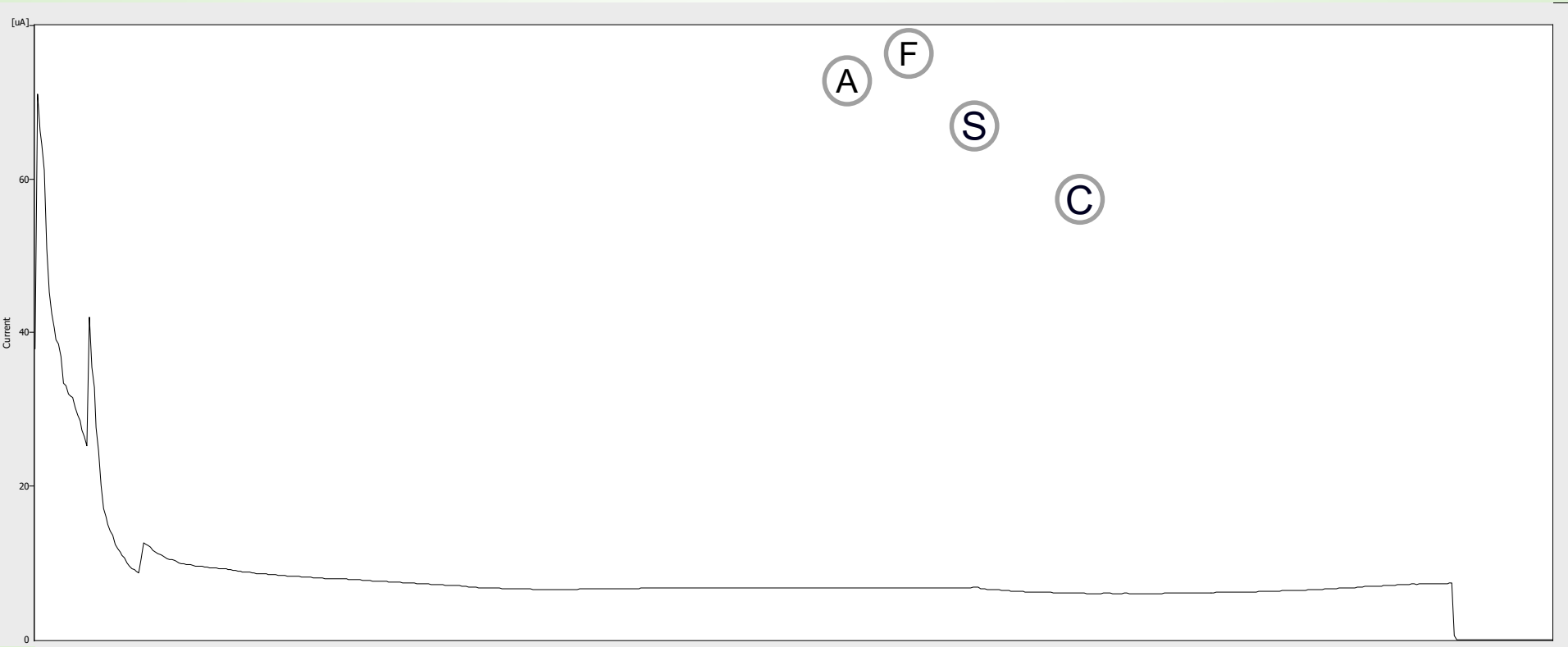
iCIEF-flow injection-MS – Hemoglobin



Focusing: 1000 V (1 min), 2000 V (1 min), 3000 V (6 min), 15 s interval time
Sample: 2% ASFC Hb, 2% AESlyte 6-9, 70% MC 0.5 %
Separation capillary **50x0.2 mm**

Mobilization: 0.120 µL/min + 3000 V (25 min), 15 s interval time
Sample: 2% ASFC Hb, 2% AESlyte 6-9, 70% MC 0.5 %

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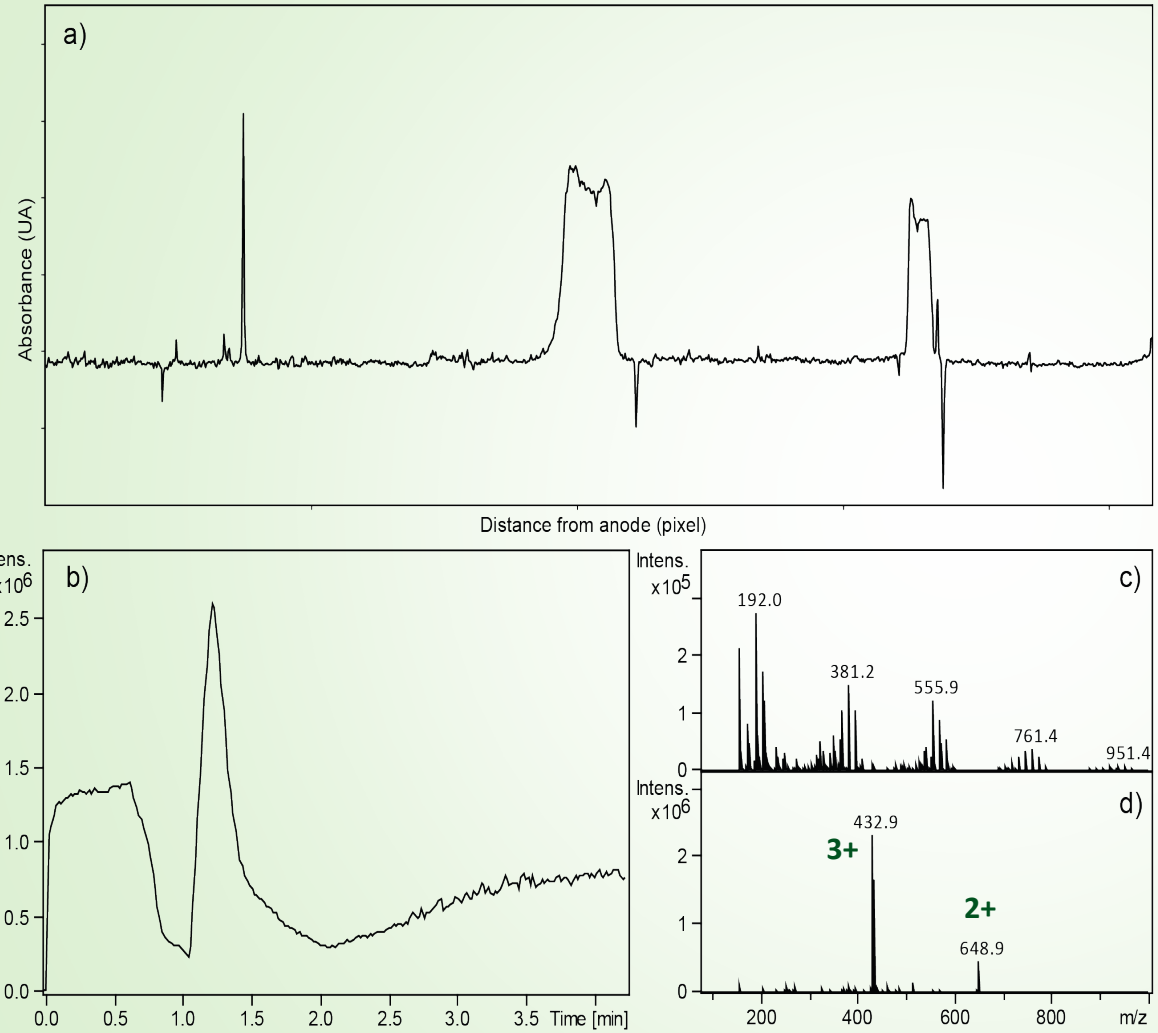
*i*CIEF of Peptide Mix with Direct Coupling



a. image after the focusing in the iCIEF
b. base peak from the cut of Angiotensin
c. spectrum cut before the peak with methylcellulose
d. spectrum of the Angiotensin peak.

Sample: 5% Angiotensin I and Leucine-enkephalin 9 (50 µg/mL), 2% AESlyte (pH 4-8), 70% methylcellulose (0.5 %). Focusing: 1kV (1 min), 2kV (1 min), 3kV (6 min), interval time 15 s. Mobilization: flow 0.120 µL/min, 3kV 11 (25 min), 15 s interval time

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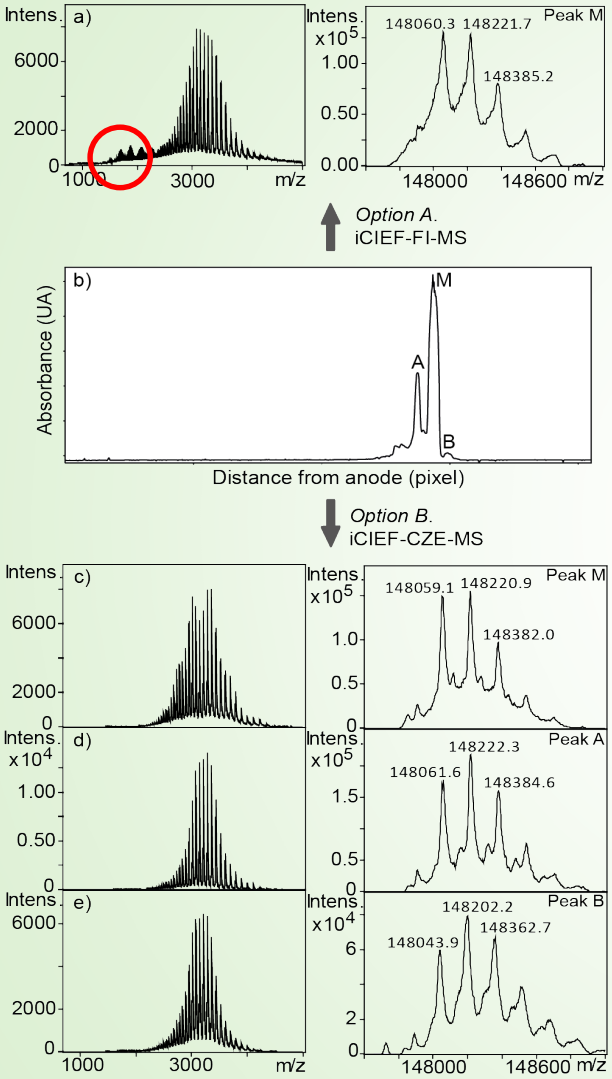
*i*CIEF of mAB coupled to CE-MS



a) Flow injection mode for the main variant M (1.25 mg/mL).
b) Example of image after focusing for the mAb in 1% AESlyte pH 16 3-10, 2% AESlyte pH 8 -10.5 HR, and 70% methylcellulose 0.5%
c) With CE-MS coupling for the main variant M (1.25 mg/mL)
d) the main acidic variant A (2.0 mg/mL) and
e) the main basic variant B (6.0 19 mg/mL)

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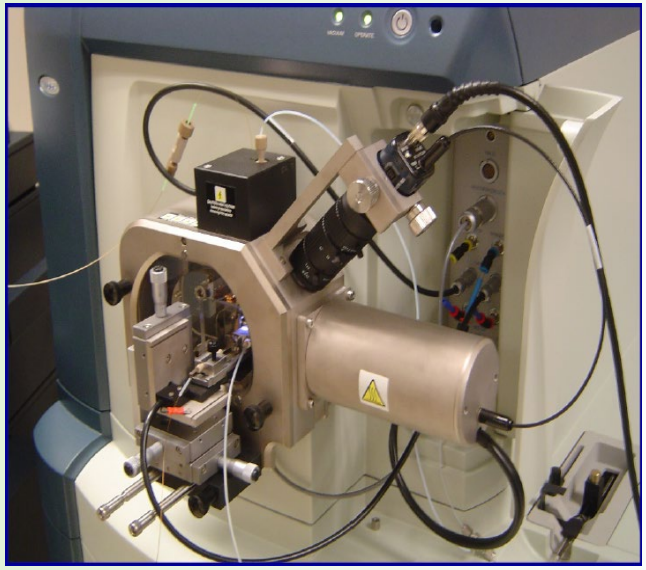
cIEF-MS Direct Coupling

Waters: NanoLockSpray-Exact-Mass-Ionization-Source



Thermo Fisher: Nanospray Flex™ Ion Sources

- *Concentration of mAbs should be between 0.5 – 3 mg/mL*
- *Add sheath liquid through T-piece*
- *Composition: 2% formic acid in 50% methanol aqueous solution*
- *Sheath liquid flow rate: 0.4 – 4 µL/min*
- *ESI voltage: 2.85 – 3.25 KV*
- *Fragmentation voltage: 40 – 180 V*

Conclusions:

- ✓ iCIEF-MS coupling is feasible
- ✓ AES CEInfinite with Prep option is an open platform solution for iCIEF-MS coupling
  - Voltages for CE-separation and ES-ionization can be decoupled allowing simply use an LC-MS nano- or µLC-sprayer
  - Use CZE as second dimension more MS friendly (background elimination) and improves peak shape
  - Direct coupling through ESI-IF using sheath solvent under development

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