Capillary Iso-Electric Focusing (CIEF) – Prime Methodology for Protein Characterization

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Gerard Rozing, *Capillary Isoelectric Focusing*, Encyclopedia of Analytical Chemistry,
https://doi.org/10.1002/9780470027318.a9731
Acknowledgement slides Gyula Vigh with permission

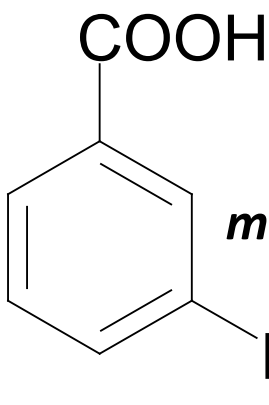
About Iso-Electric Focusing

Iso-Electric Focusing

Separation of amphoteric molecules.

Are molecules that carry both, ionizable acidic and basic functional groups

E.g., amino acids, peptides, proteins, other molecules (not zwitter ions)

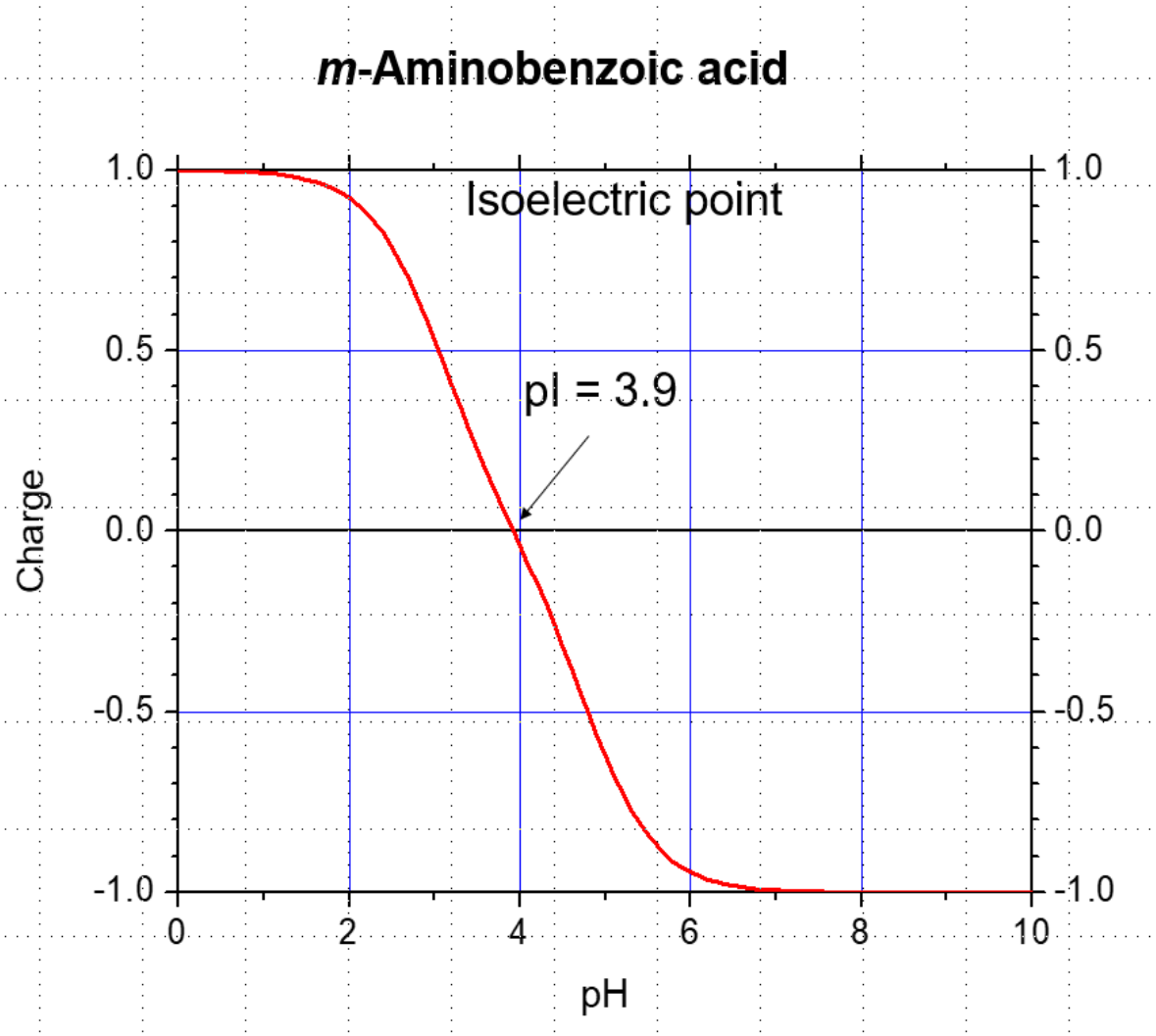


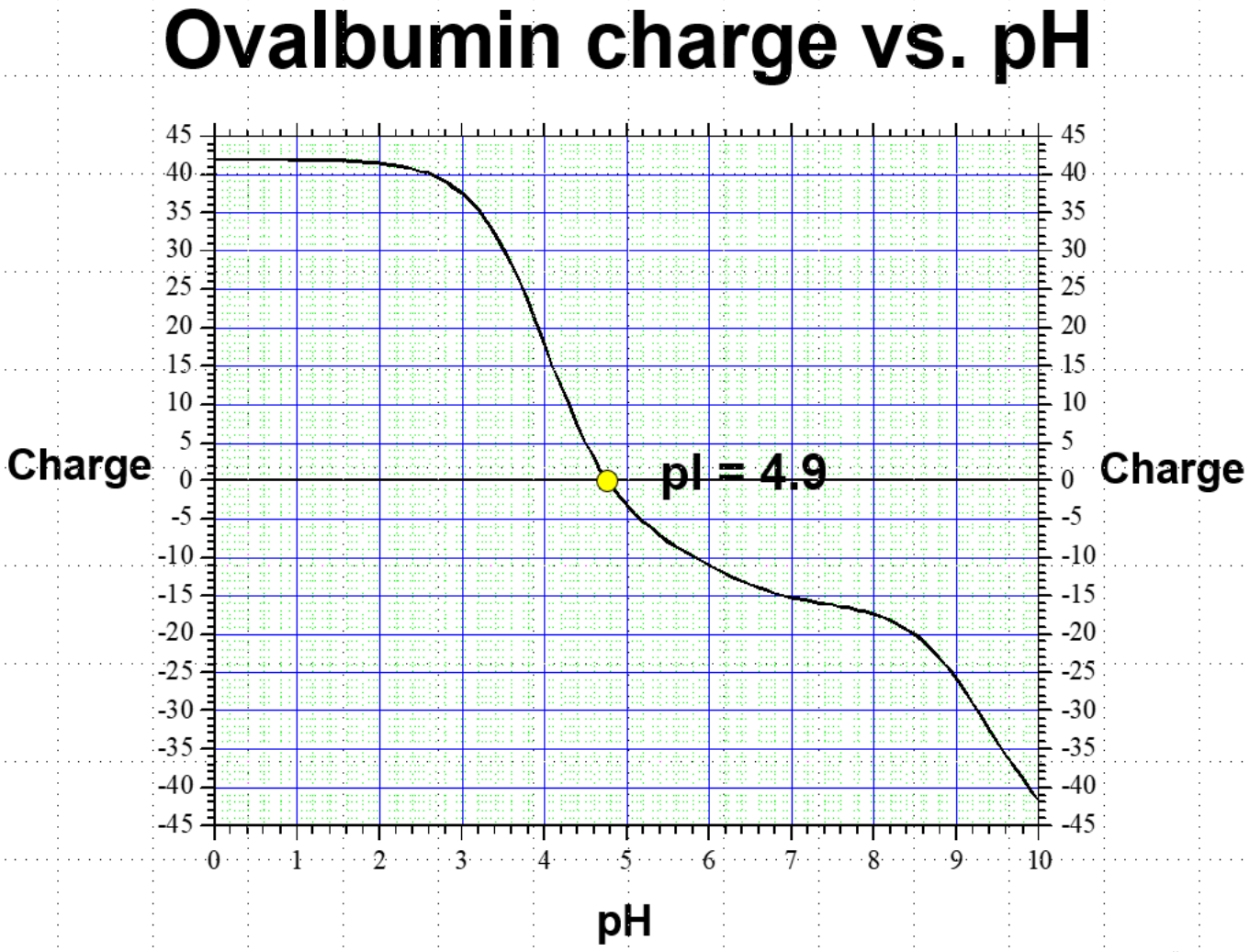
$pK_a = 3.08$

***m*-aminobenzoic acid**

$pK_b = 4.76$

Iso-electric Point



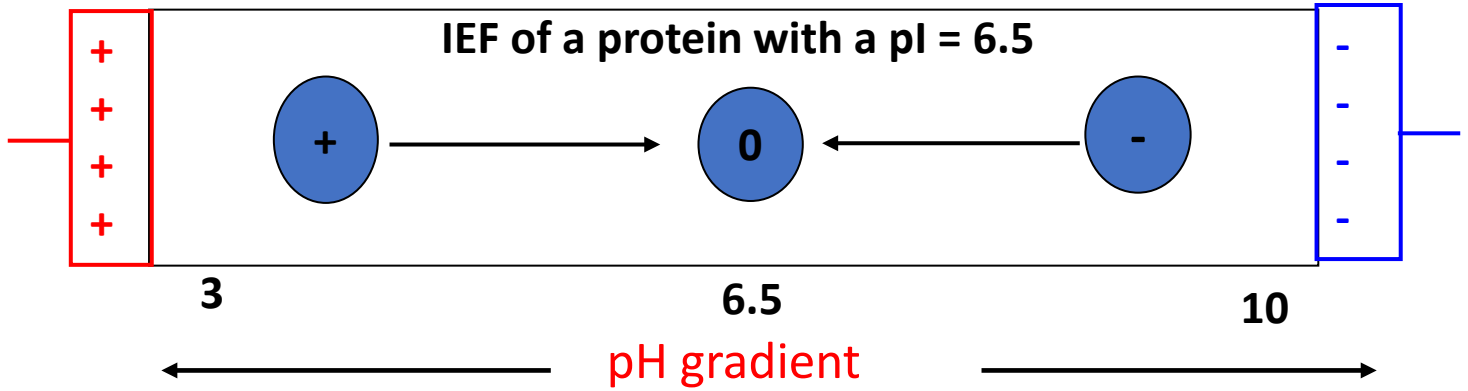


pI calculation for peptides and proteins
http://calistry.org/calculate/isoelectric-point-calculator

Isoelectric Focusing

Rilbe (Svensson): Theoretical Aspects of IEF*

- In slab gel IEF, so called carrier ampholyte molecules become sorted according to their isoelectric point (pI) and from low to high pI → pH gradient.
- At the location where pH equals the pI of the carrier ampholyte, it has no net charge and no mobility in the electric field. Zone dispersion by diffusion is counteracted by the electrical force on the molecule.



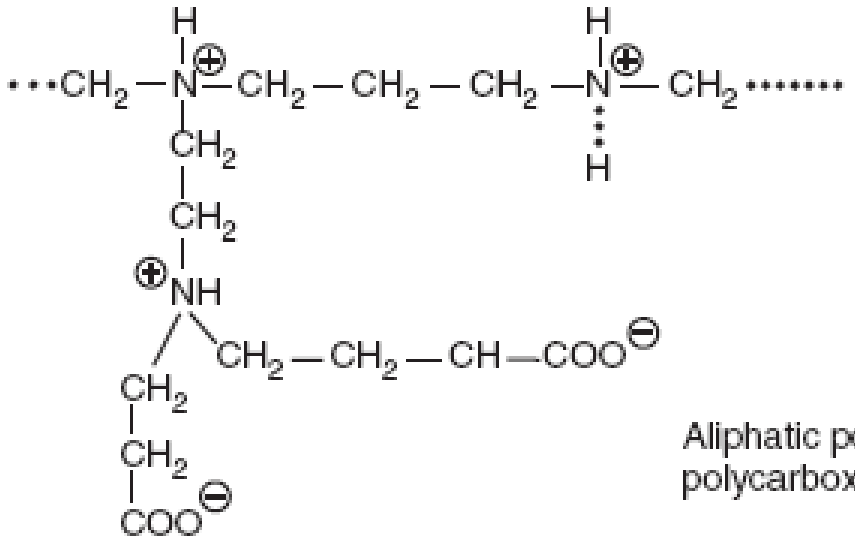
formed by focusing of CARRIER AMPHOLYTES in an electric field

Annals of the New York Academy of Sciences 209, 11 1973

Carrier Ampholytes*

Are oligomers formed by the condensation of poly amines with unsaturated carboxylic acids:

- molecular weight between 300 – 1000
- high buffering capacity and conductivity at their pI
- In an ampholyte formulation of 1000 to 10 000 CA species in 3 < pI < 10 range

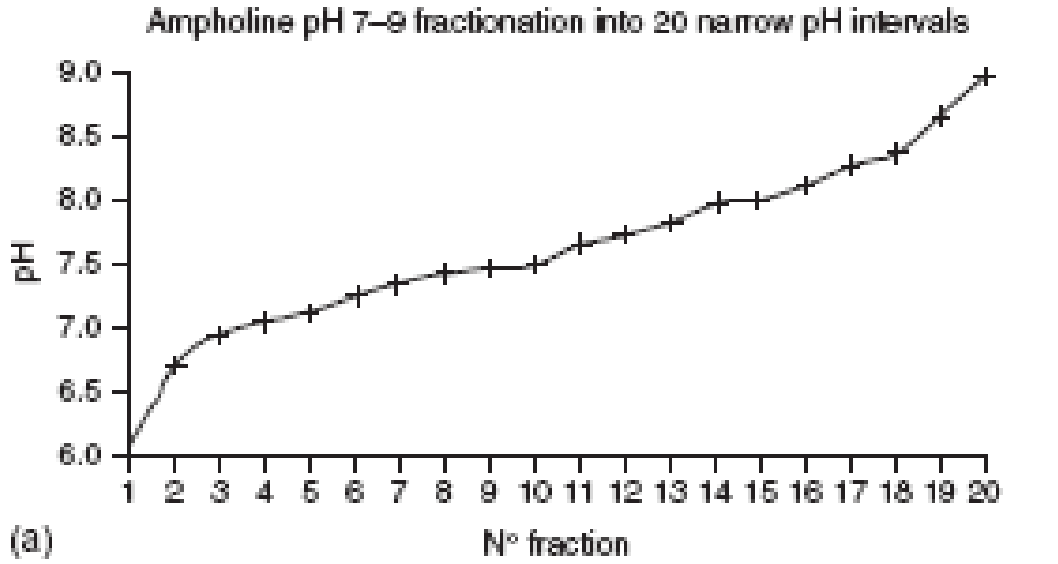


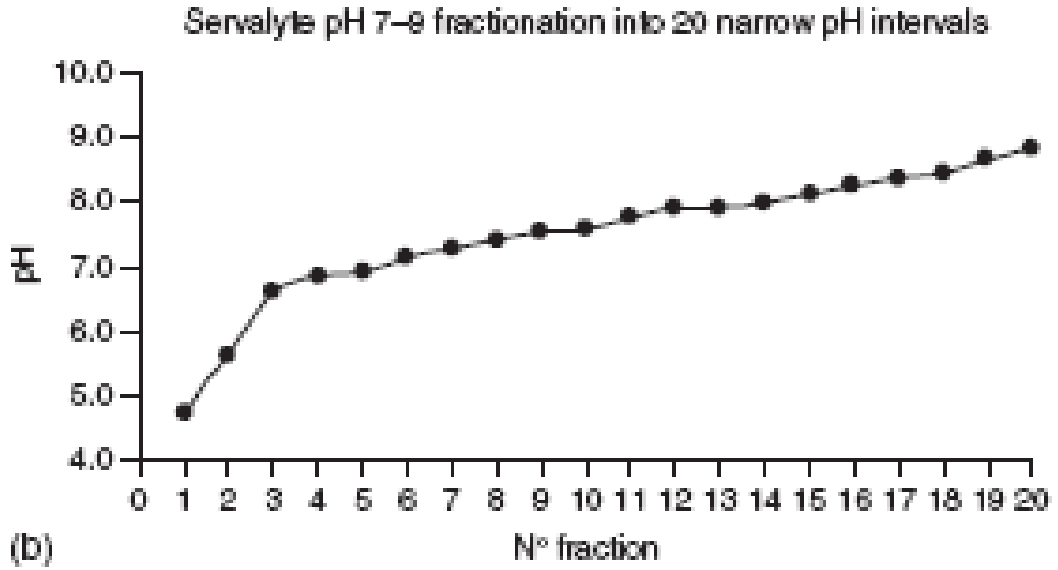
Aliphatic polyamino polycarboxylic acids

Sources of ampholytes, slide 12

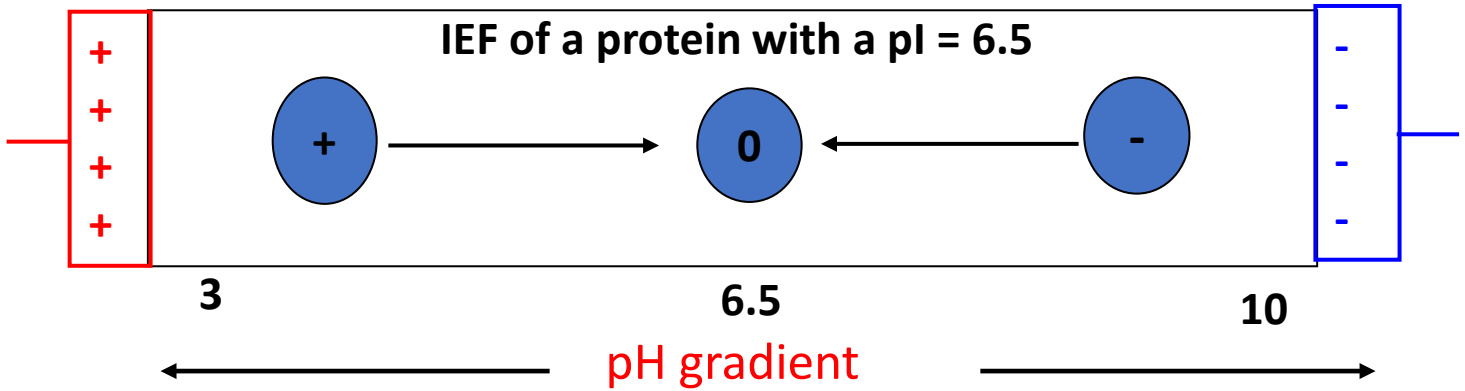
Establish pH-gradient

Fractionation of pH gradient for two ampholytes according to Sebastiano et al, Electrophoresis, 27, 3919 (2006)





Isoelectric Focusing - Formats



Homogeneous mixture of CA and an anticonvective polymer (PAA) on a plate
Flanking channels with low pH solution (anolyte) and high pH solution (catholyte)
Immobilized pH Gradient (IPG) carrier ampholytes chemically attached to the polymer
Laborious methodology

From slab gel IEF to capillary IEF

Slab gel IEF:

- **Separation in space**
- **pH gradient formation and protein separation separate steps**
- **Distinct sample application**
- **Zone visualization by staining after separation**

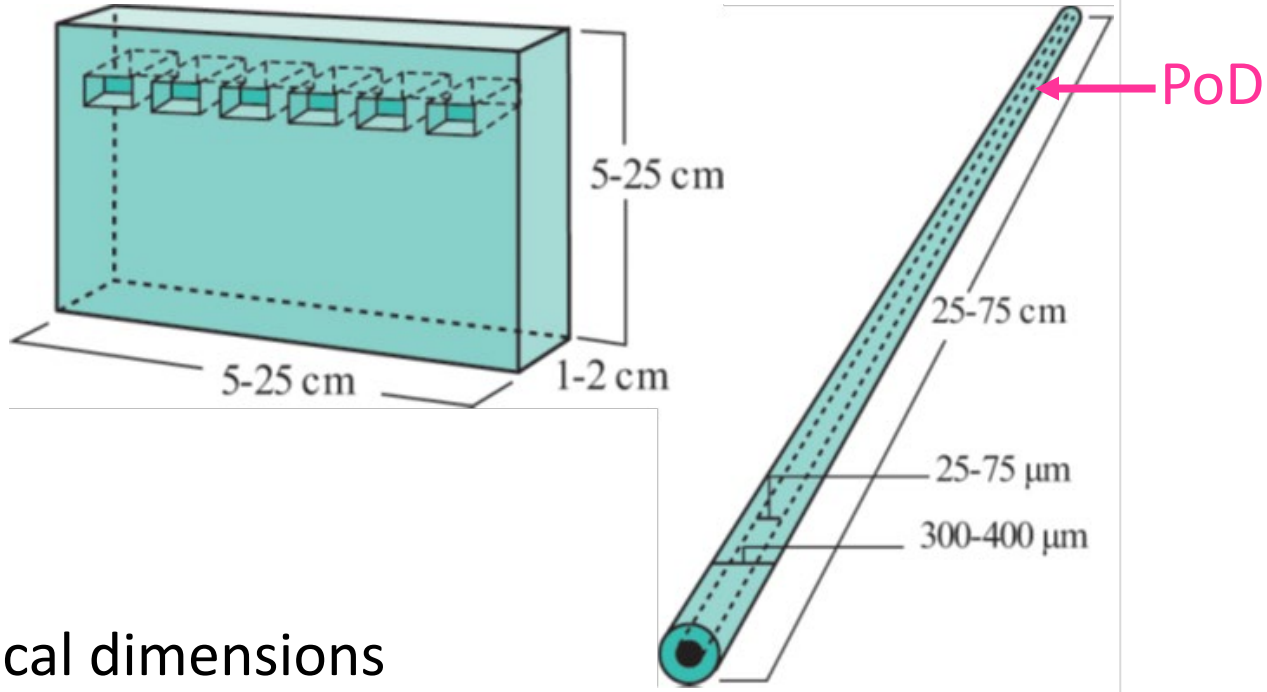
Capillary IEF

- **Separation in time**
- **pH gradient formation, sample application and protein separation must take place concurrently**
- **Make sure that focusing of the sample compounds occurs before the point of detection**
- **Focused zones are stagnant**
- **The zones must be mobilized to pass by point of detection.**

Change Separation Format

spatial separation

temporal separation



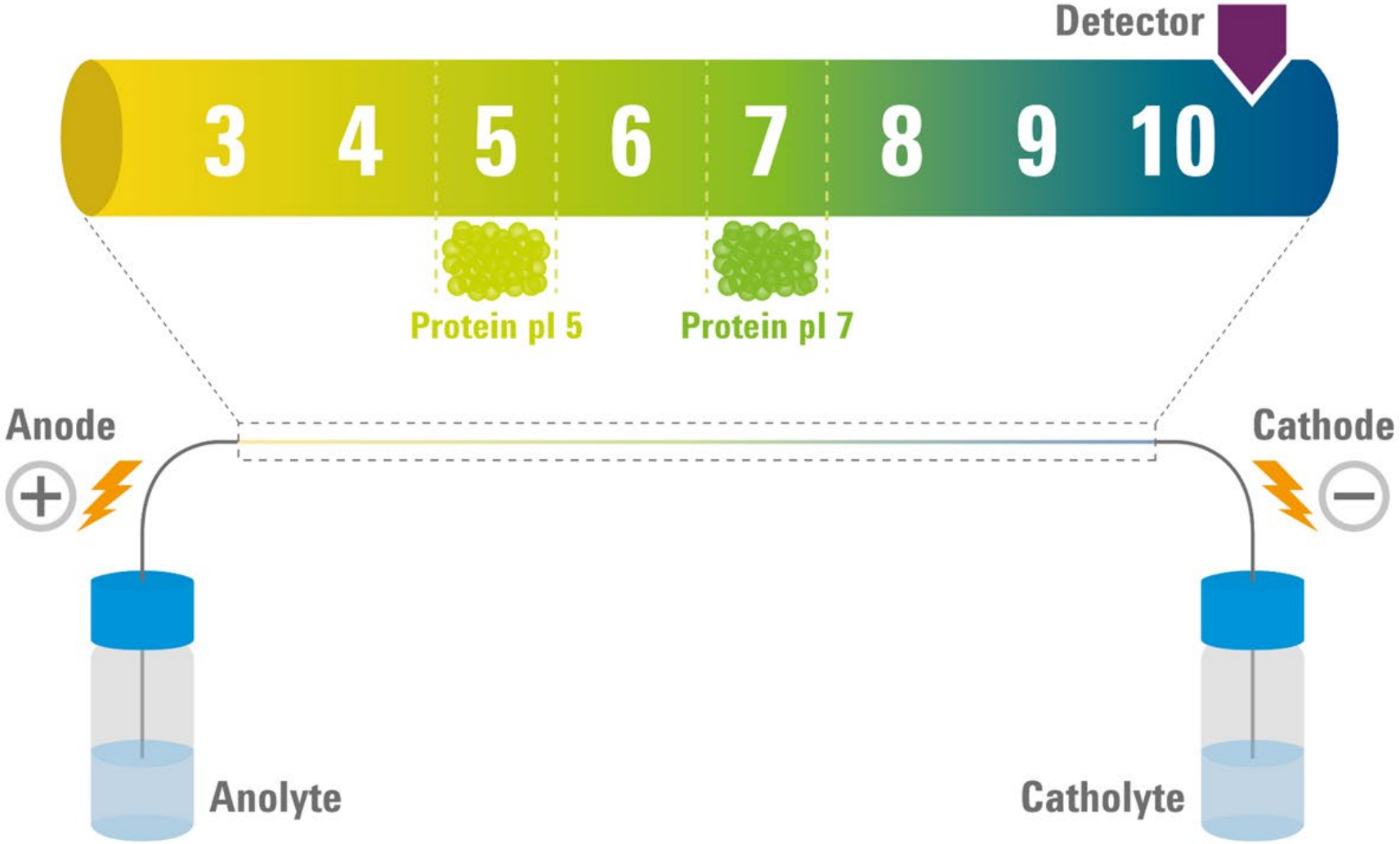
Typical dimensions

Capillary Iso-Electric Focusing (CIEF)

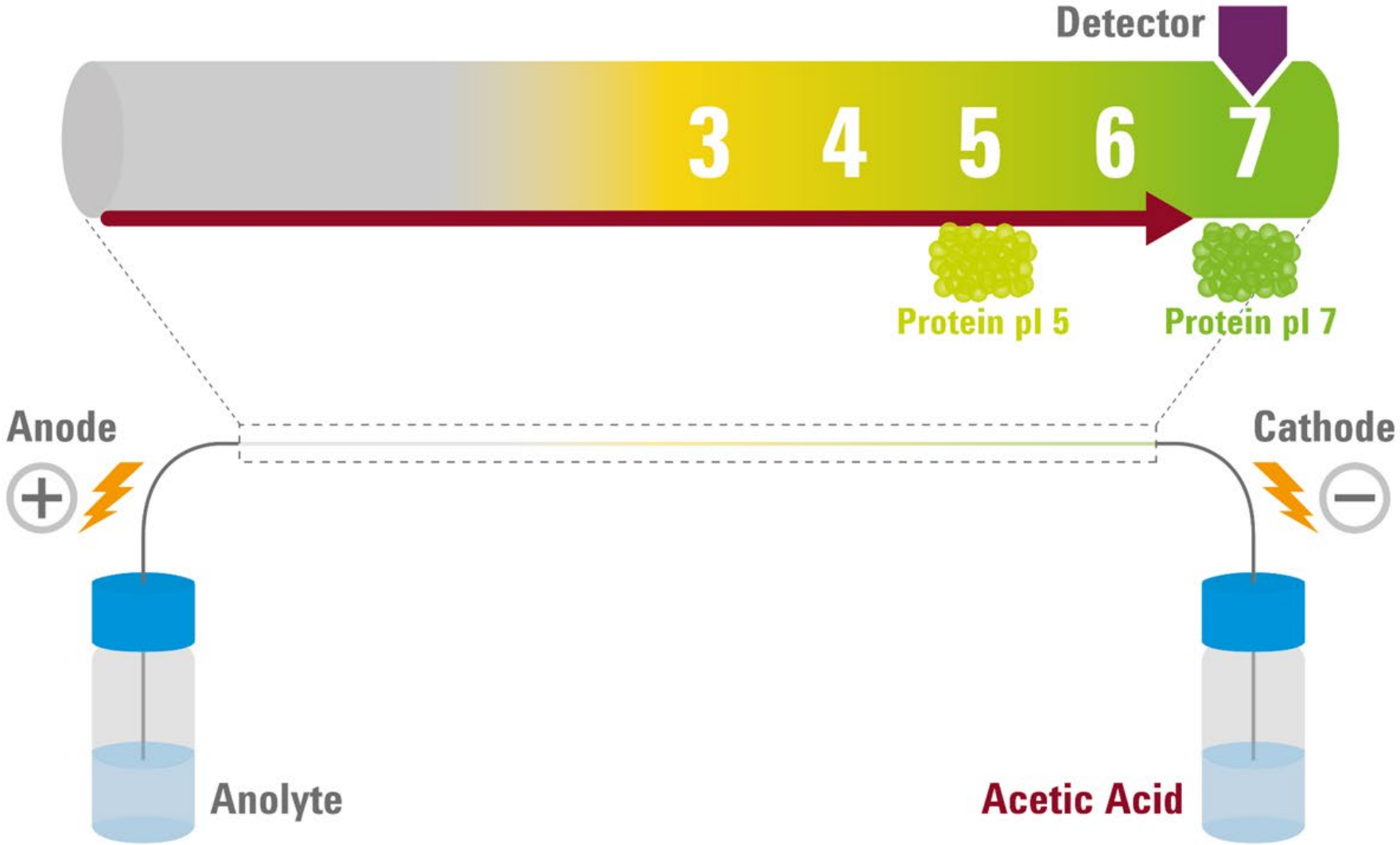
At the beginning of a CIEF run, the whole capillary (coated fused silica) is filled with:

- Carrier Ampholytes (CA) not shown
- Protein or peptide sample to be analyzed (amphoteric molecules)
- pI markers (amphoteric molecules) not shown
- Low and high pI sacrificing ampholytes (blockers) to keep the focused zones within the capillary and before POD
- Additives that suppress residual EOF and improve solubility of focused protein zones
- The inlet side low pH (anolyte,H_3PO_4), high positive voltage, anode
- The outlet side high pH (catholyte, NaOH), grounded, cathode

Capillary Iso-Electric Focusing (CIEF)



Capillary Iso-Electric Focusing (CIEF)



CIEF Practice – Carrier Ampholytes

Table 1 Overview of commercially available ampholytes Products in green are originally manufactured

Name	pI range	Manufacturer	Website	Suppliers
Ampholine		LKB/Amersham	https://www.sigmaaldrich.com/life-science/biochemicals.html	Discontinued
Pharmalyte®	Narrow and wide-range 8 part numbers	GE Healthcare	https://www.sigmaaldrich.com/life-science/biochemicals.html	Merck KGaA, Darmstadt, Germany
BioLyte®	Narrow and wide-range 13 part numbers	BIO-RAD, Life Science, Hercules, California, USA	https://www.bio-rad.com/en-de/product/ampholytes	BIO-RAD
AESlyte	Narrow and wide-range 16 part numbers	Advanced Electrophoresis Solutions, Cambridge, Ontario, Canada	https://ceinfinite.com/	AES Ltd., Cambridge, ON, Canada Isogen Life Science, Utrecht, The Netherlands
Servalyt™	Narrow and wide-range 24 part numbers	SERVA Electrophoresis GmbHHeidelberg, Germany	https://www.serva.de/enDE/Catalog/186_Electrophoresis_Isoelectric_Focusing_0_195.html	Worldwide distributors
Sinulyte™	Narrow and wide-range 23 part numbers	SINUS Biochemistry & Electrophoresis GmbH, Heidelberg Germany	https://www.sinus-biochem.de/index.html	Alibaba, China. Koma Biotech, Korea
Sepalyte™	Narrow and wide-range 23 part numbers	ProTec Bioseparation	http://www.protec-biosep.de/	ProTec Bioseparation UG. & Co.KG, Heidelberg, Germany
Roti®lyte	Narrow and wide-range 13 part numbers	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	https://www.carlroth.com/en/en/	Worldwide distributors
Zoom™ Carrier Ampholytes	Narrow and wide-range 5 part numbers	Invitrogen	https://www.thermofisher.com/de/en/home/brands/invitrogen.html	Thermo Fisher Scientific

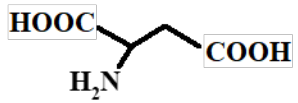
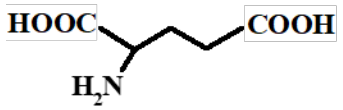
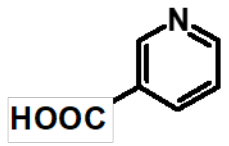
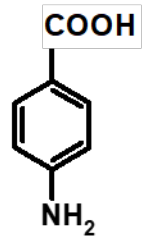
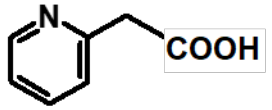
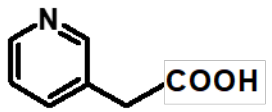
CIEF Practice - Capillaries

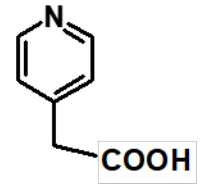
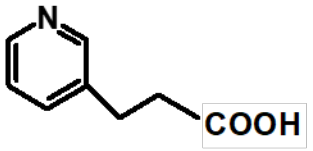
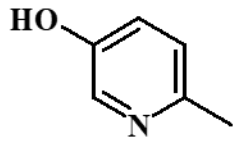
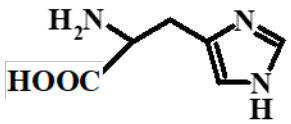
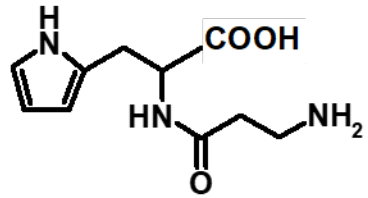
Table 3 Capillaries for CIEF

Supplier	Chemistry	pH range	Format	Website
Agilent Technologies	µSIL-FC, nonpolar fluorocarbon (FC) polymer	2.5– 10	Precut lengths with detector window, 50 and 75 µm	https://www.agilent.com/cs/library/catalogs/public/5991-5623EN.pdf
	µSIL-DB-1, nonpolar x-linked dimethylpolysiloxane		Bulk packing	
	µSIL-DB-17, nonpolar x-linked dimethylpolysiloxane		Bulk packing	
	Polyvinyl alcohol (PVA), hydrophilic polymer	2.5–9.5	Precut lengths with detector window, 50 and 100 µm	
Sciex (Fisher Scientific)	eCAP neutral capillary, hydrophilic N-substituted polyacrylamide, US Patent US005370777A	2–9	Length 67 cm, I.D. 50 µm, with detector window	https://www.fishersci.com/shop/products/e-capillaries/nc9829116 https://de-store.sciex.com/de/EUR/capillary-electrophoresis
Micro Solv Technology Corporation	Controlled EOF and zero EOF Coated/bonded capillaries for CE and cIEF	2–8.5	Length 1 m, I.D. 50 and 75 µm	https://mtc-usa.com/faqce100sa_ti

CIEF Practice – pI markers

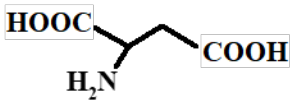
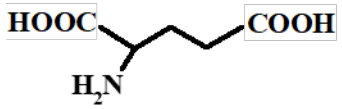
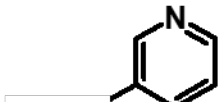
- pI markers are amphoteric molecules
- Preferably of simple structure
- UV light (260 nm) absorption
- Focus in a small zone
- Measured pI ≠ true pI

Compound	pI
	2.7
	3.2
	3.4
	3.6
	4.2
	4.3

Compound	pI
	4.4
	4.8
	7.2
	7.6
	8.1

CIEF Practice – pI markers

- pI markers are amphoteric molecules
- Simple stricture
- UV light (260nm) absorption
- Focus in a small zone

Compound	pI
	2.7
	3.2
	3.4

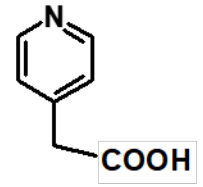
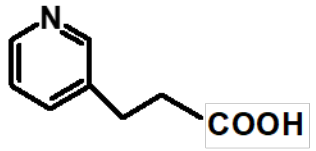
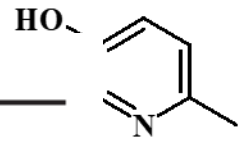
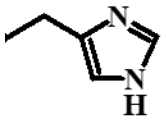
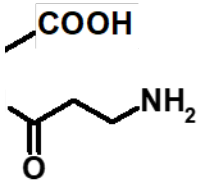
Compound	pI
	4.4
	4.8
	7.2
	7.6
	8.1

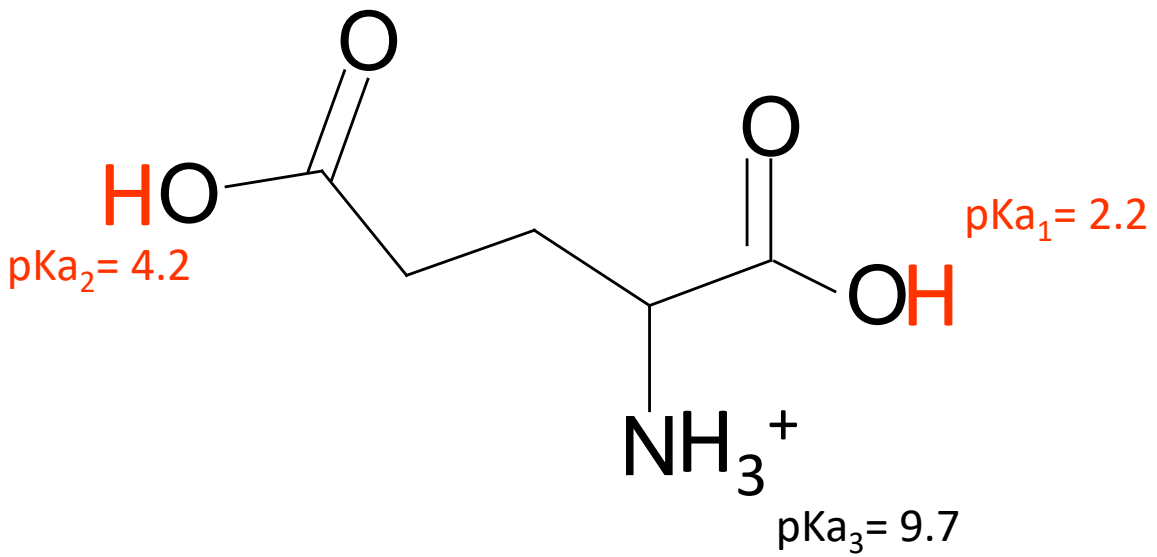
Table 4 Commercial sources of pI markers

Source	Products	Chemistry	Website
Sigma-Aldrich	16 marker, pI 3.0–10.5	Fluorescent, low-MW amphoteric compounds	https://www.sigmaaldrich.com/germany.html
Sciex separations	cIEF peptide marker kit, pI 4.1, pI 5.5, pI 7.0, pI 9.5, pI 10.0	Synthetic peptide	https://de-store.sciex.com/de/EUR/capillary-electrophoresis/reagents-chemistries-and-kits/chemistries
Advanced electrophoresis solutions	Reference standards for iCIEF, CIEF, and IEF	Low-MW nonpeptide amphoteric compounds	https://ceinfinite.com/referencestandards/
Protein simple	23 markers, pI 2.85–10.45	n.a.	https://www.proteinsimple.com/consumables_ice.html
Bio-Rad	Biomark™, 17 pI markers for CIEF, pI 5.6–10.4	Synthetic	https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_9606.pdf

CIEF Practice – Blockers

Name	pI
IDA	2.3
ASP	2.9
GLU	3.2
HIS	7.4
CARN	8.2
LYS	9.7
ARG	10.5

Glutamic acid, pI 3.2



Capillary Iso-Electric Focusing Instrumentation

Advantages

- Can be done on commercial CE instruments
- Provides automation, data acquisition quantitation and results documentation

Disadvantages

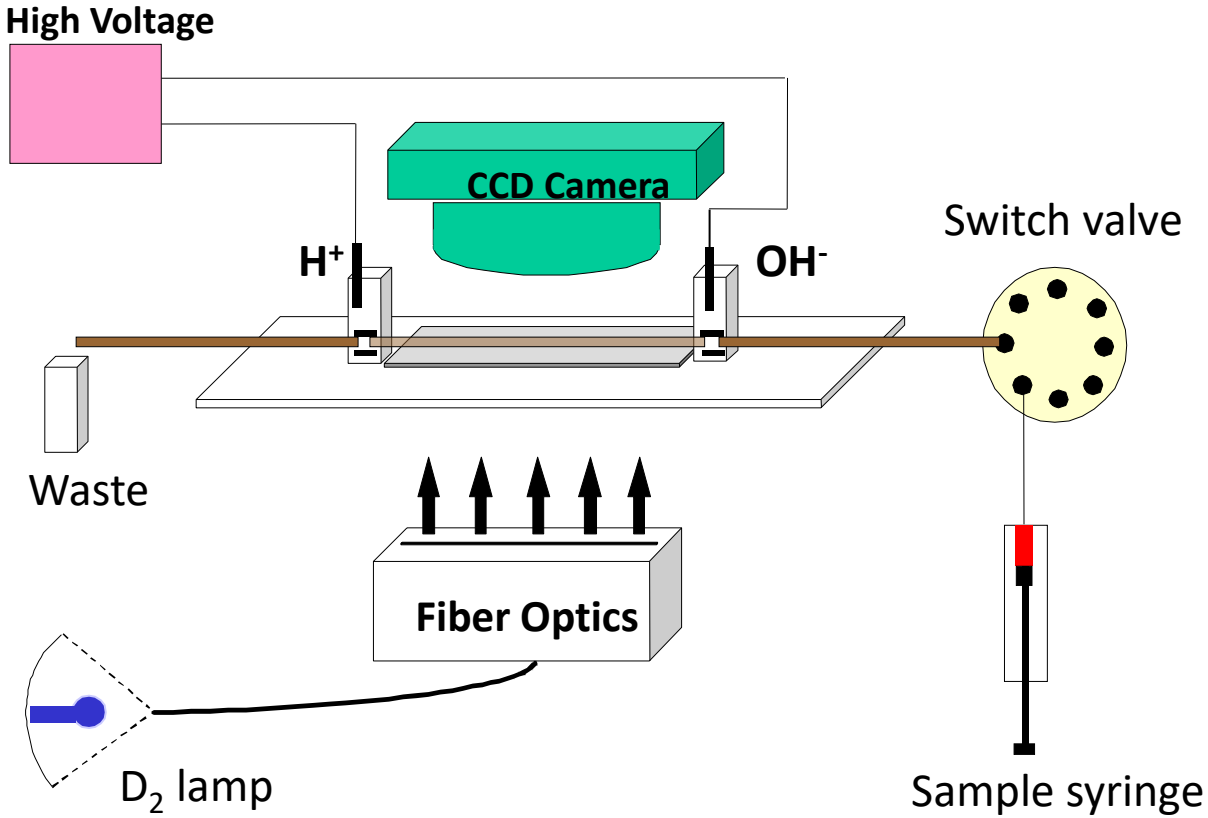
- Long analysis time (about 30-60 min.)
- Long method development time (days)
- **Blind to the focusing process**
- Non-relevant peaks detected
- Low resolution due to mobilization process

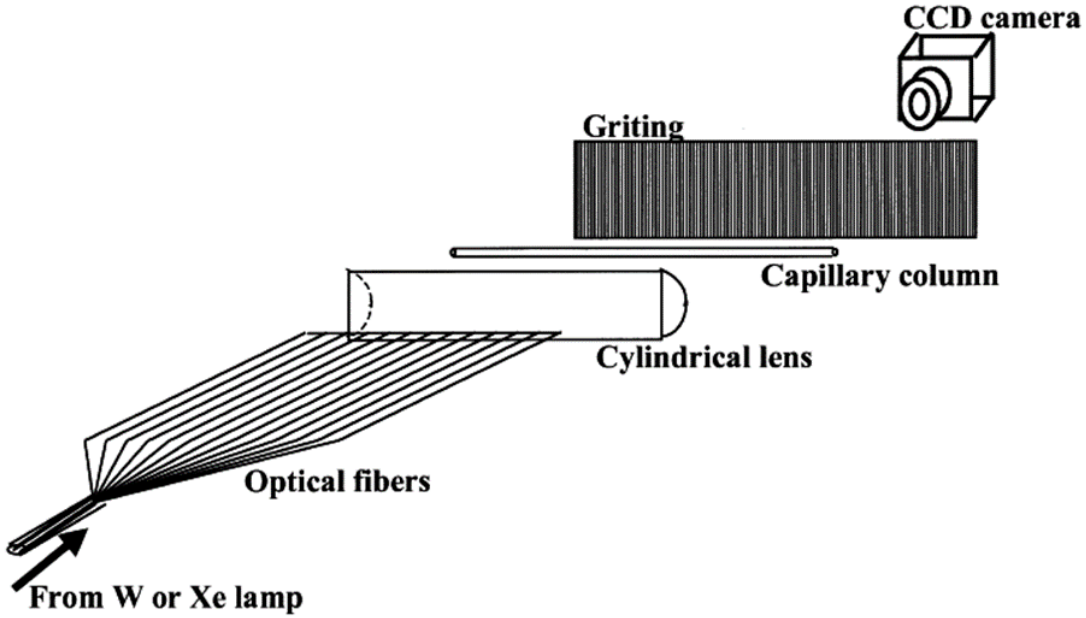
PA 800 Plus
Sciex, Danaher Corporation

G7100, Agilent Technologies

Whole Column Image Detection (iCIEF)

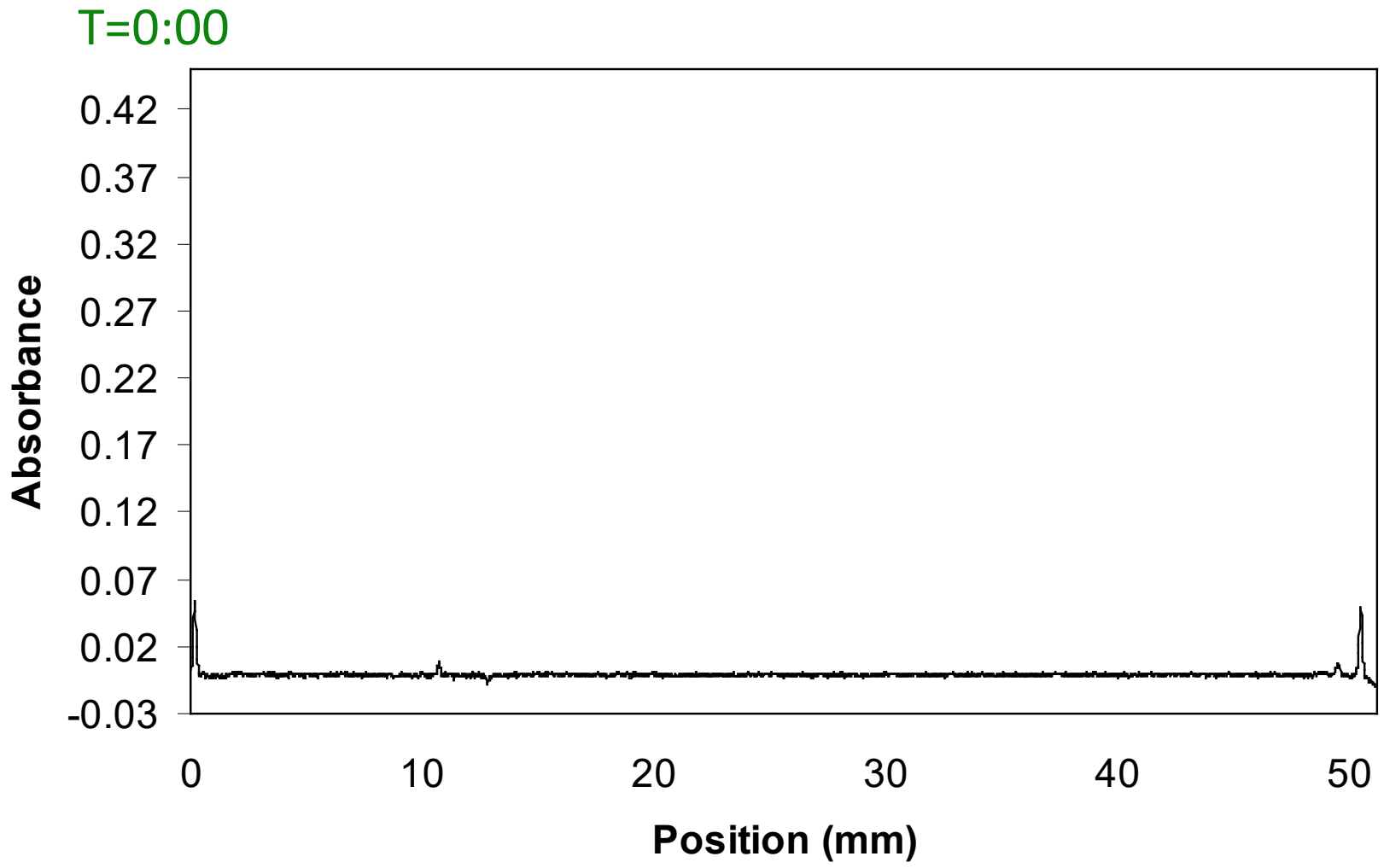
- First described by Pawliszyn and Wu,
- Commercialized by Convergent Bioscience in 2000, iCE280.
- Acquired by Protein Simple (now Bio-Techne) in 2010.
- Established market leader, iCE3 and iCE Maurice





Wu, J, Pawliszyn, J. et al. „*Anal. Chem., 1992, 64, 224-227*
Analytica Chimica Acta, 383, 67–78 (1999)

Development of IEF Separation of five pI Markers recorded with whole column imaging detector



Development of IEF Separation of five pI Markers

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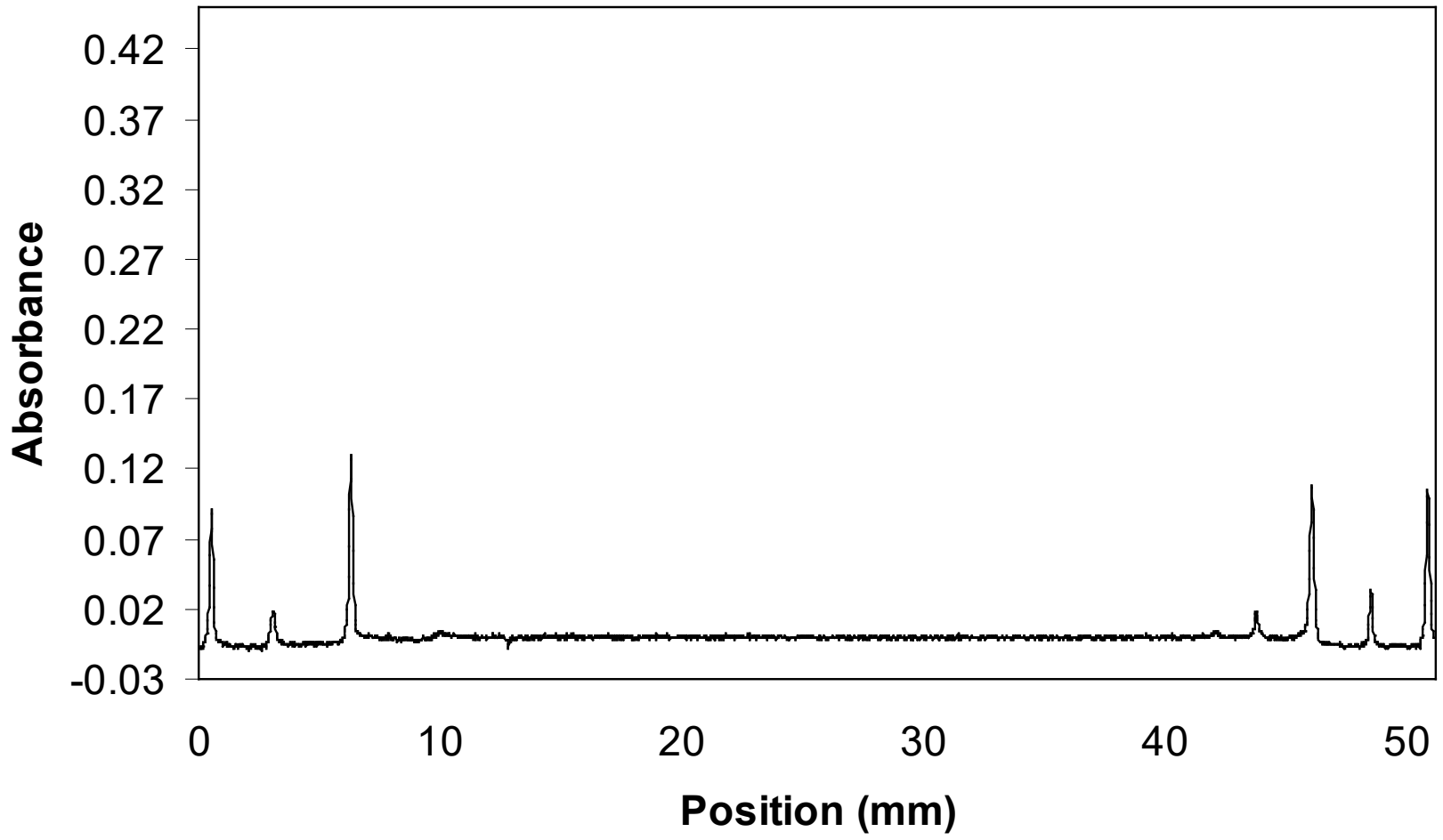
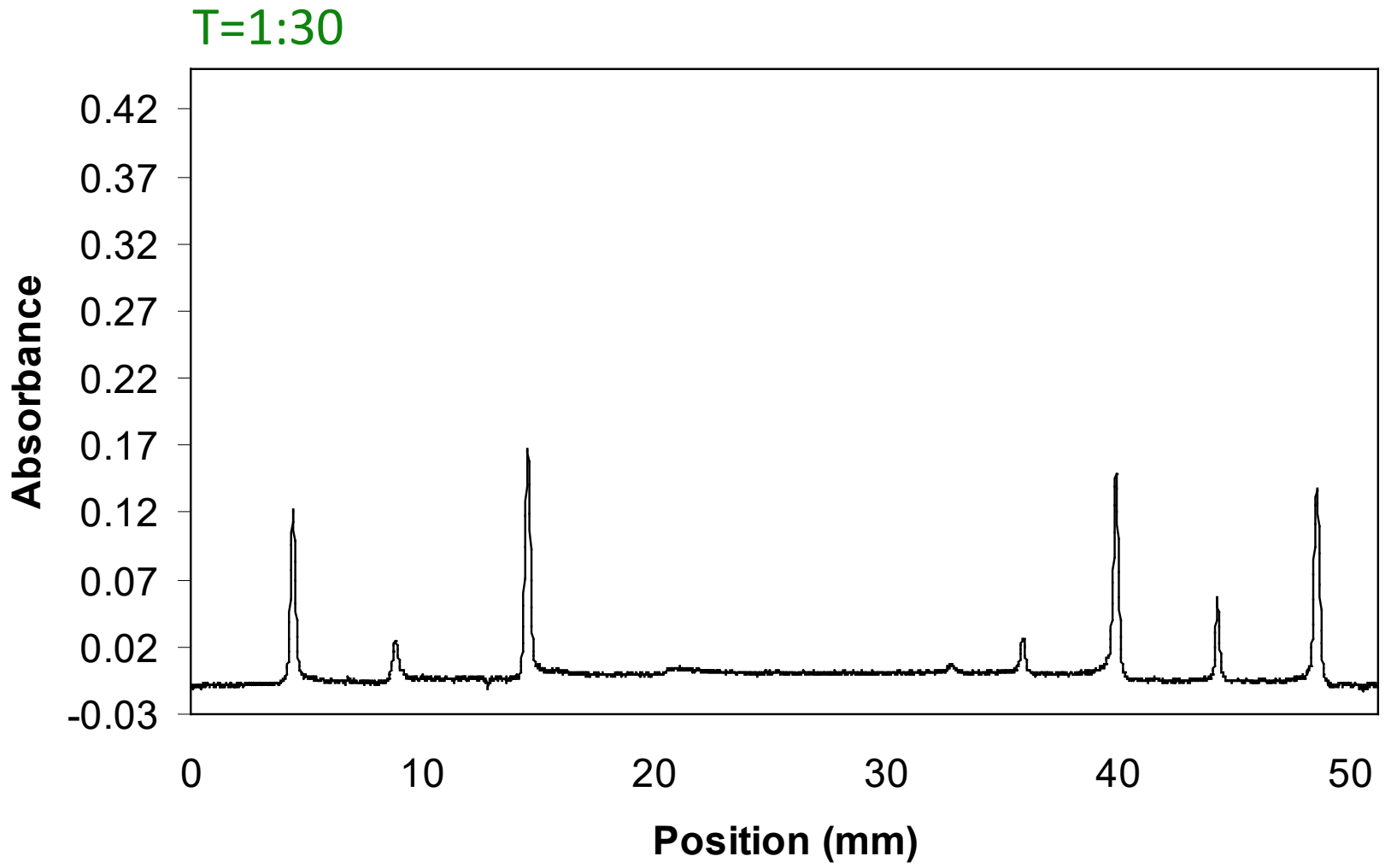
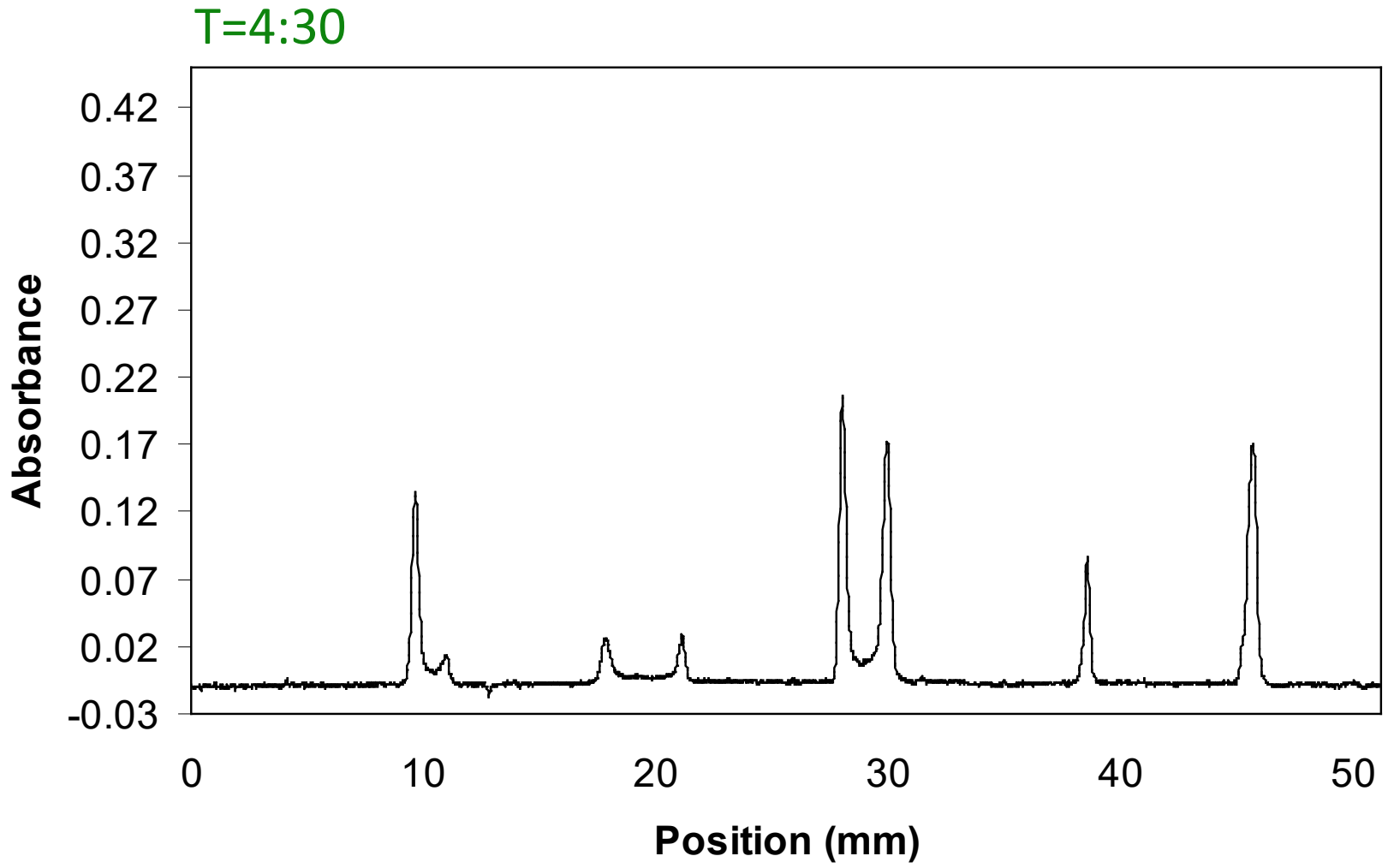


Development of IEF Separation of five pI Markers



Development of IEF Separation of five pI Markers



Development of IEF Separation of five pI Markers

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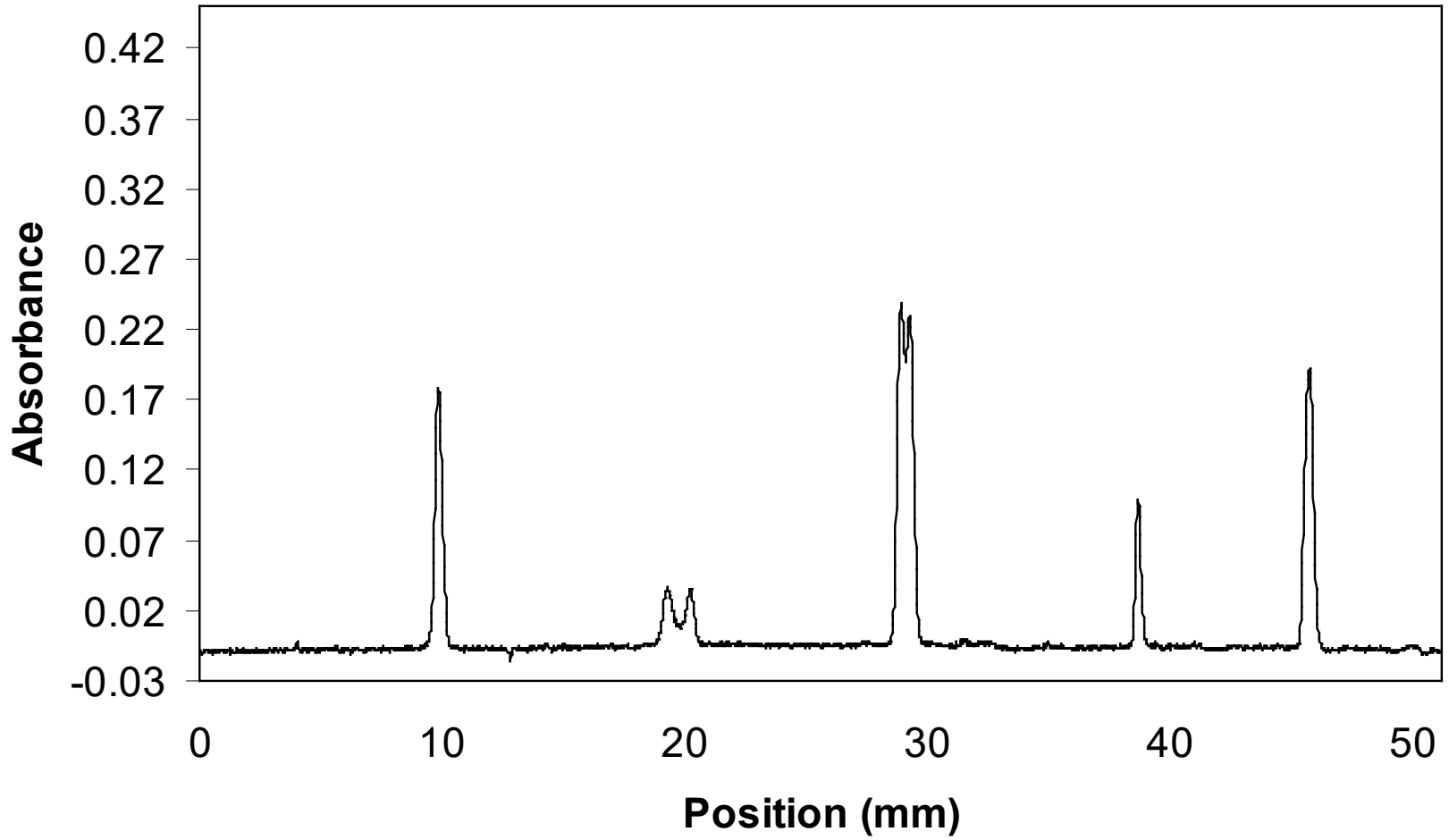
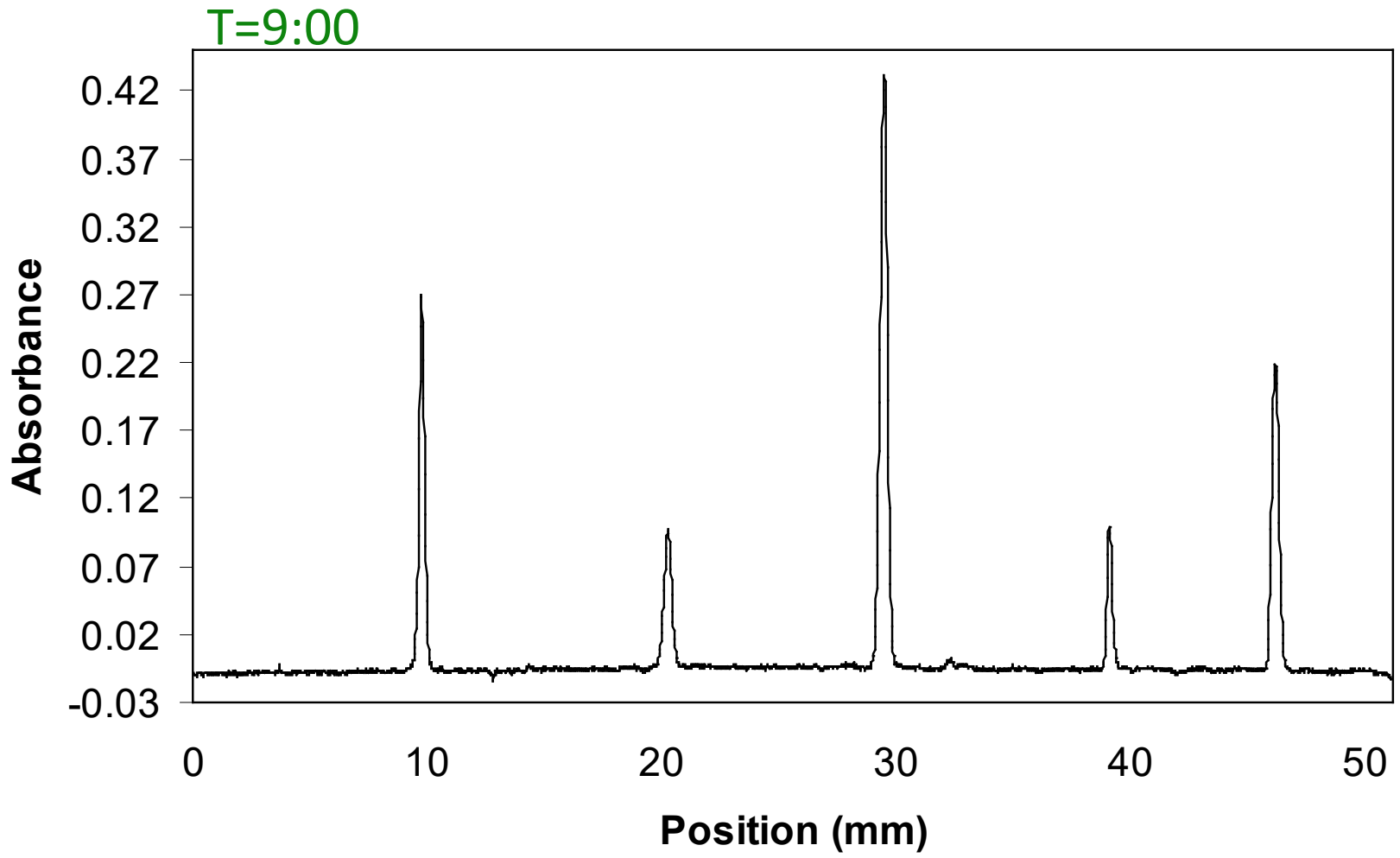


Development of Separation of five pI Markers



Separation of Hemoglobin Isoforms

AESlyte pH 3-10
Protein Simple Hemoglobin Standard
Marker 3.4, 9.5, Focus. Time 3’
Separation Capillary 50x0.1 mm

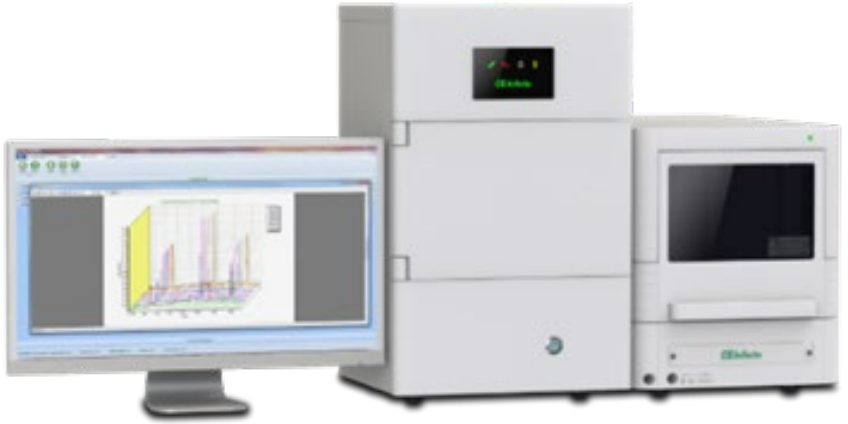
CIEF

iCIEF

PA 800 Plus
Sciex, Danaher Corporation

G7100,
Agilent Technologies

iCE Maurice,
Bio-Techne



CEInfinite,
Advanced Electrophoresis Solutions

Assessment of CIEF vs. *i*CIEF

Capillary Iso-Electric Focusing

- Standard open-platform CE Equipment: multiple CE methods
- Long cycle time (sample introduction, focusing, and mobilization)
- Long method development time
- Competence and experience required especially for troubleshooting and diagnosis
- Protein concentration >0.05mg/mL

Imaged Capillary Iso-Electric Focusing

- ✓ Dedicated CIEF system: lean and optimal for CIEF
- ✓ Short cycle time
- ✓ Rapid method development
- ✓ Easy-to-use by online monitoring, diagnose
- ✓ Protein concentration > 0.1mg/ml (iCE Maurice with fluorescence detection, 10x better)

New Developments in CIEF and iCIEF

Coupling with mass spectrometry

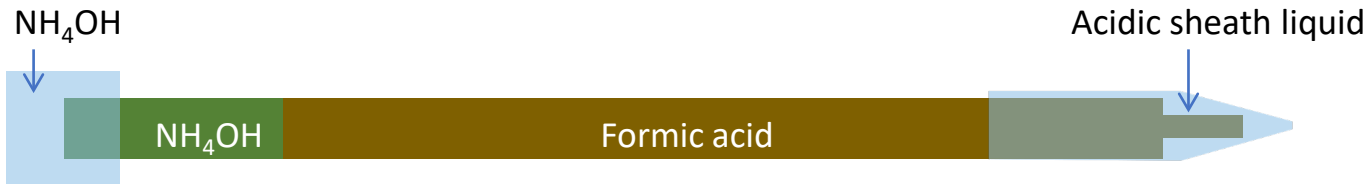
Preparative CIEF separations

Challenges in Coupling CIEF with ESI-MS

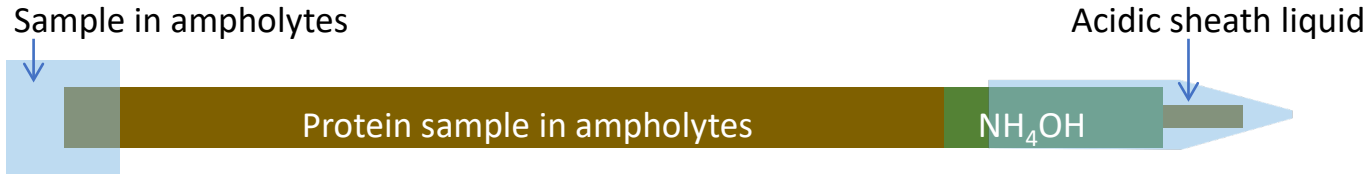
- Transport a separated/discrete small zone to the ESI interface
  - Chemical Mobilization ??
  - Pressure Mobilization → zone dispersion
- Interference/ion suppression of the ESI process by ampholytes, catholyte (high pH) and other additives
- Presence of non-volatile additives →contamination of the MS inlet
- Deal with two high voltage sources on one liquid conductor

CIEF – MS Coupling

- Step 1: inject NH_4OH plug



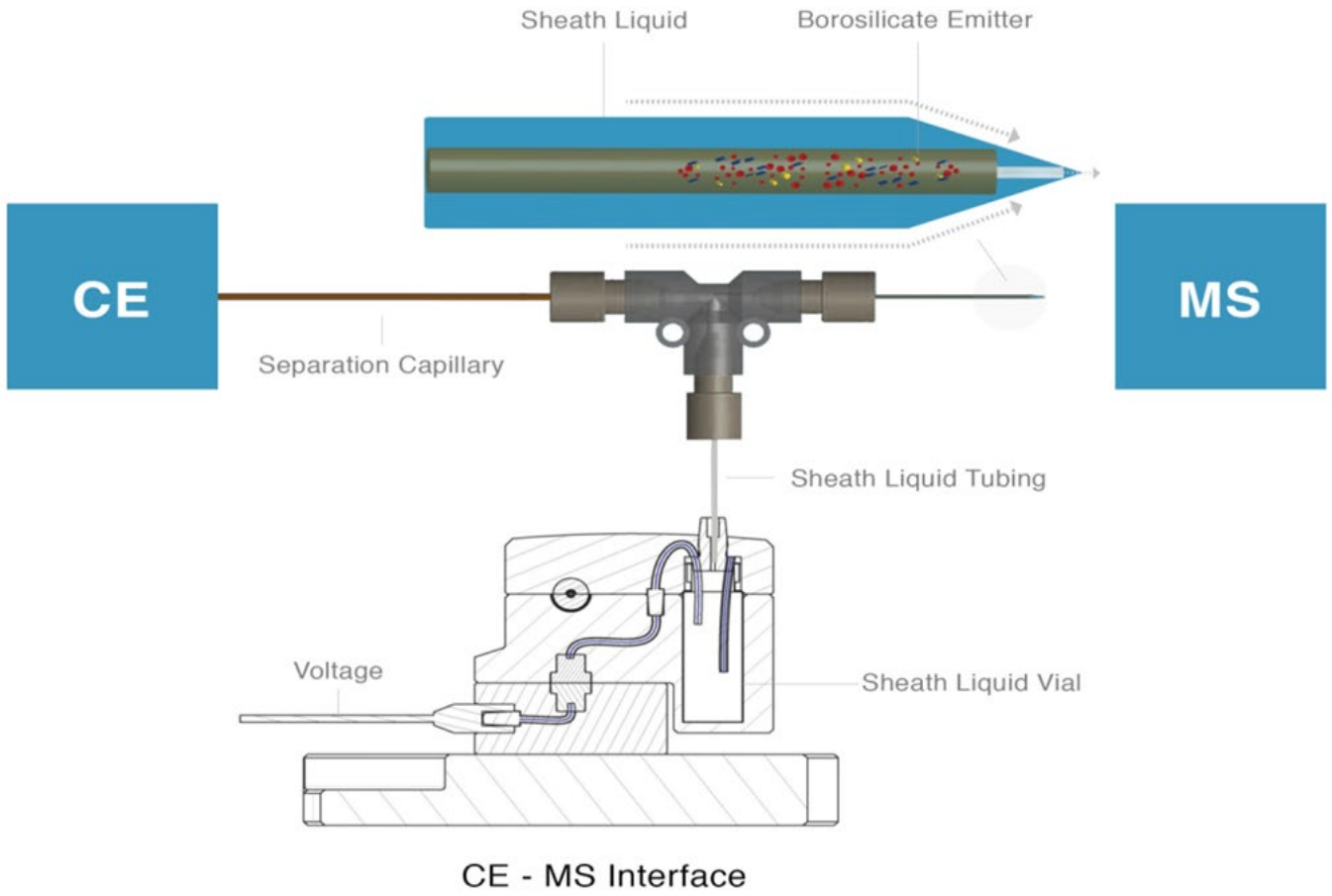
- Step 2: inject protein sample plug (in ampholytes).



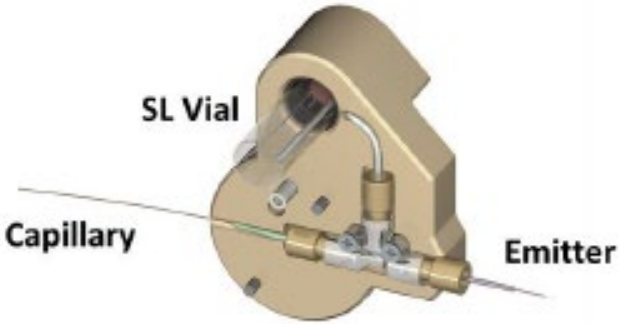
- Step 3: focusing / mobilization.

J. Dai, Q. Xia et al. CIEF-Mass Spectrometry Method for the Separation and Online Characterization of Intact Monoclonal Antibody Charge Variant, Anal. Chem. 2018, 90, 2246−2
C. Crosnier de Lassichère, Q. Xia and H. Nicar, Poster@ MSB2020
Q. Xia and J. Meixner, Agilent AppNote, February 2019, 5994-0672EN

CIEF – MS Coupling



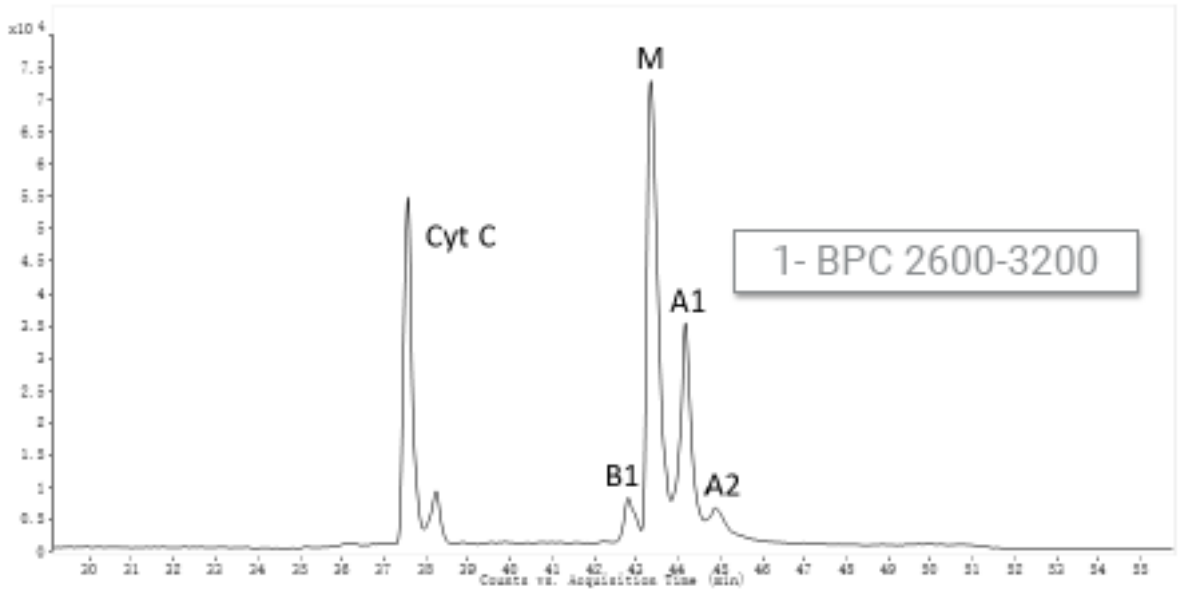
CE - MS Interface

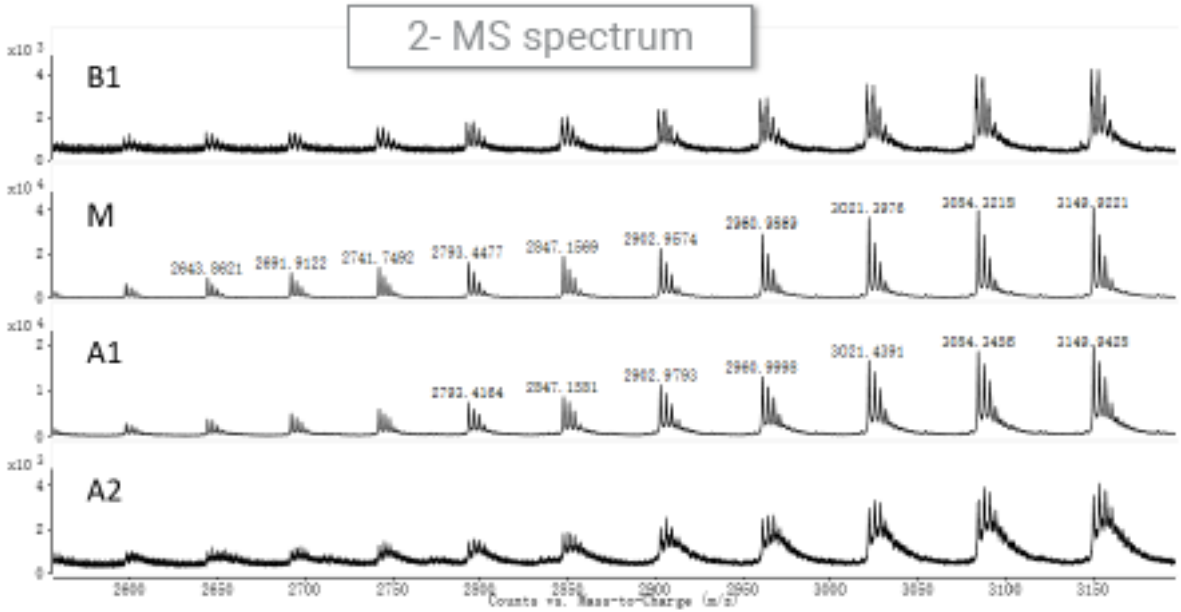


C. Crosnier de Lassichère, Q. Xia and H. Nicar, Poster@ MSB2020
Q. Xia and J. Meixner, Agilent AppNote, February 2019, 5994-0672EN

CMP Scientific, Brooklyn, NY 11226
tech@cmpscientific.com

CIEF – MS Coupling



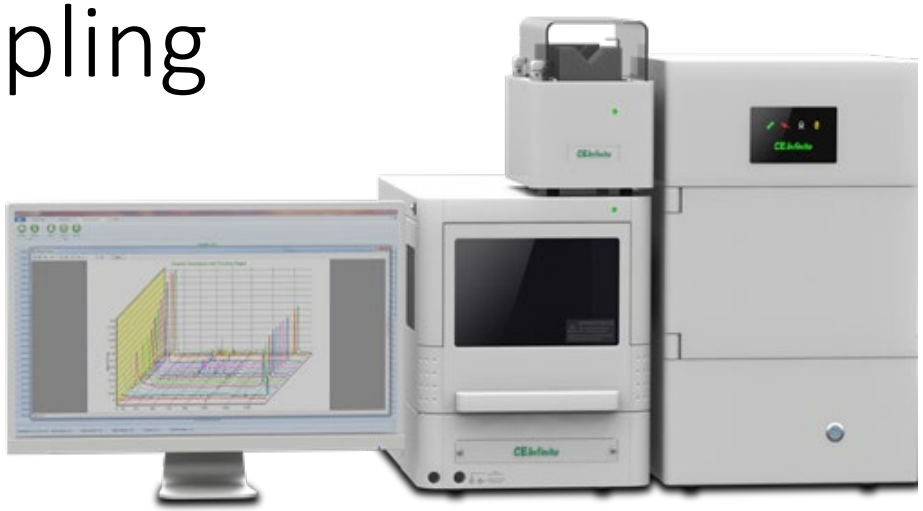


Fully automated online charge variants analysis of a mAb molecule is repeated for 100 injections After normalization with an internal pI marker (Cyt-C), the migration time of the charge variants show RSDs within 7%

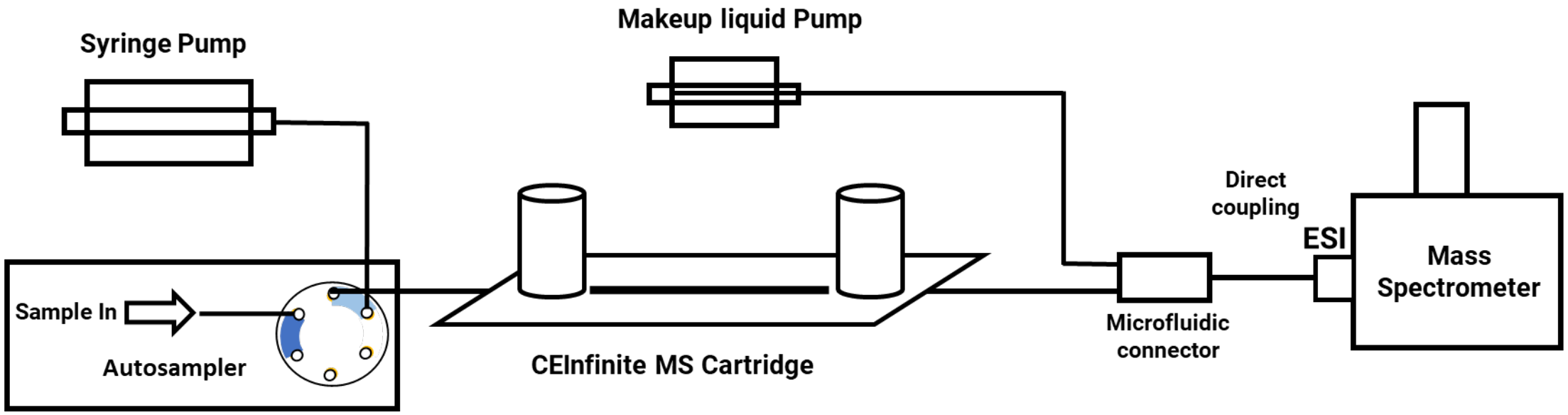
Agilent
Trusted Answers

CMPScientific

iCIEF – MS Coupling



CEInfinite Preparative CIEF

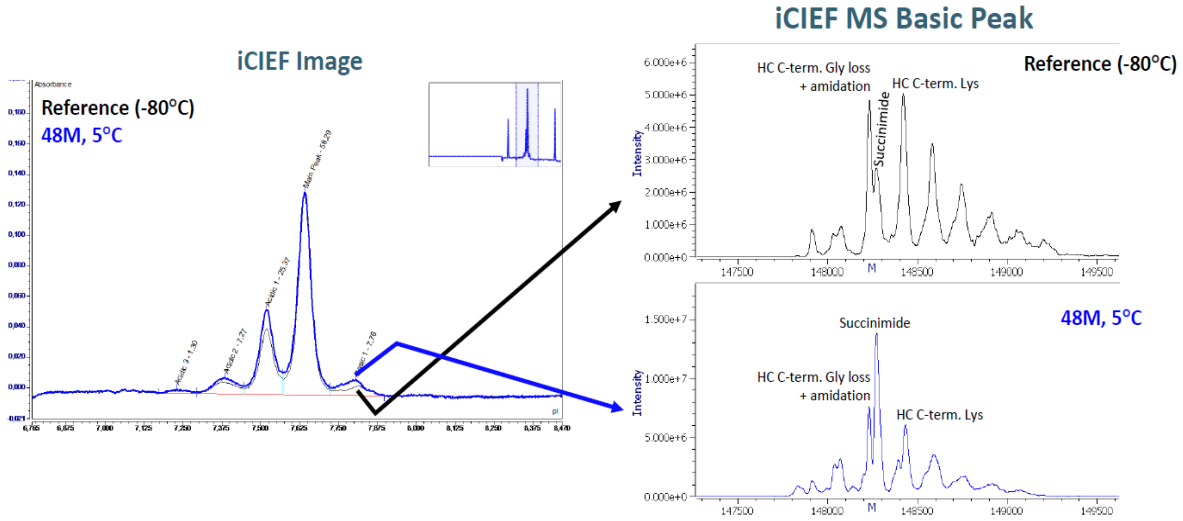


iCIEF-MS Platform

ADVANCED ELECTROPHORESIS SOLUTIONS LTD.

ThermoFisher SCIENTIFIC

The combination of iCIEF separation and HRAM Orbitrap MS provides confident insight into subtle changes in stability



Testimonial

"iCIEF plays a critical role for monitoring charge variants during biopharmaceutical development. The CEInfinite allows us to get a mass readout for the product variants detected by iCIEF, and this is a great help for understanding the chemical nature of the charge variants.

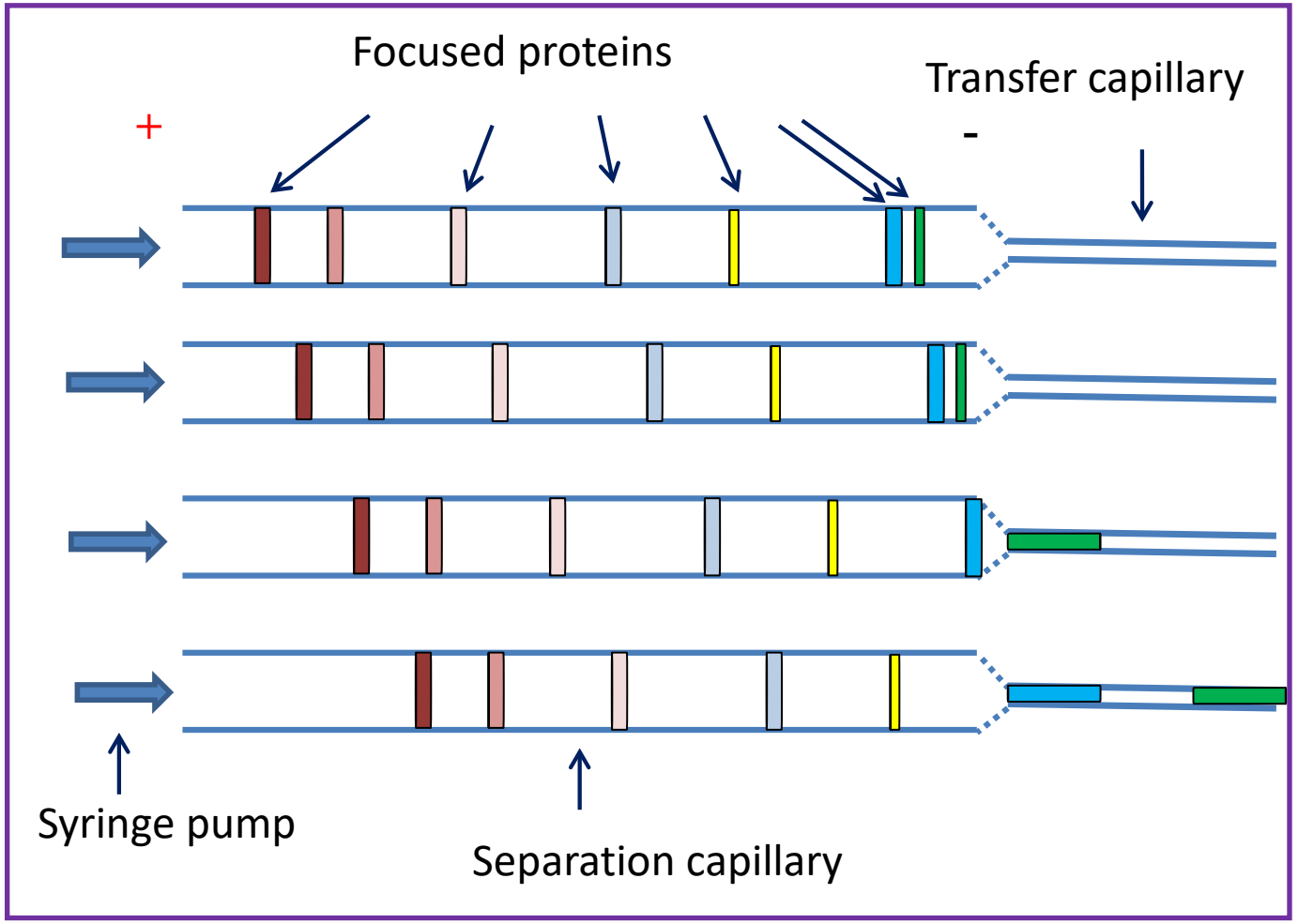
When combining the CEInfinite with high resolution accurate mass MS provided by Orbitrap MS systems, detailed insight into product variants and product stability can be achieved.

In short, iCIEF-MS using the CEInfinite is a very powerful characterization tool in biopharmaceutical development."

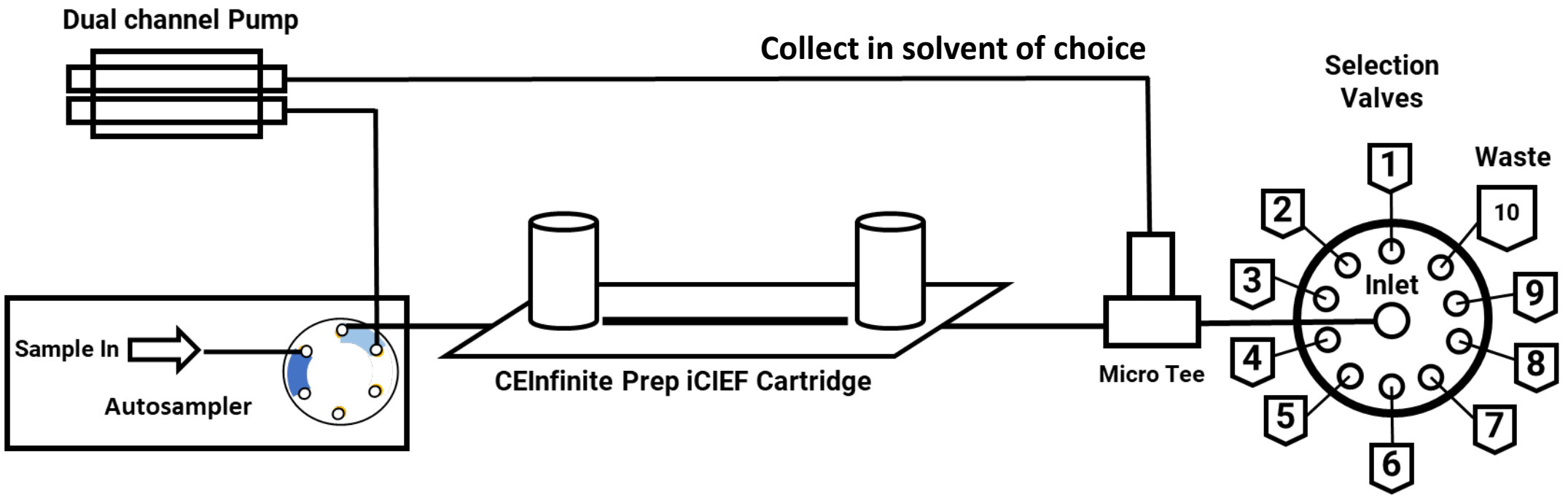
Dan Bach Kristensen, Ph.D.
Principal Scientist

symphogen
a Servier Company

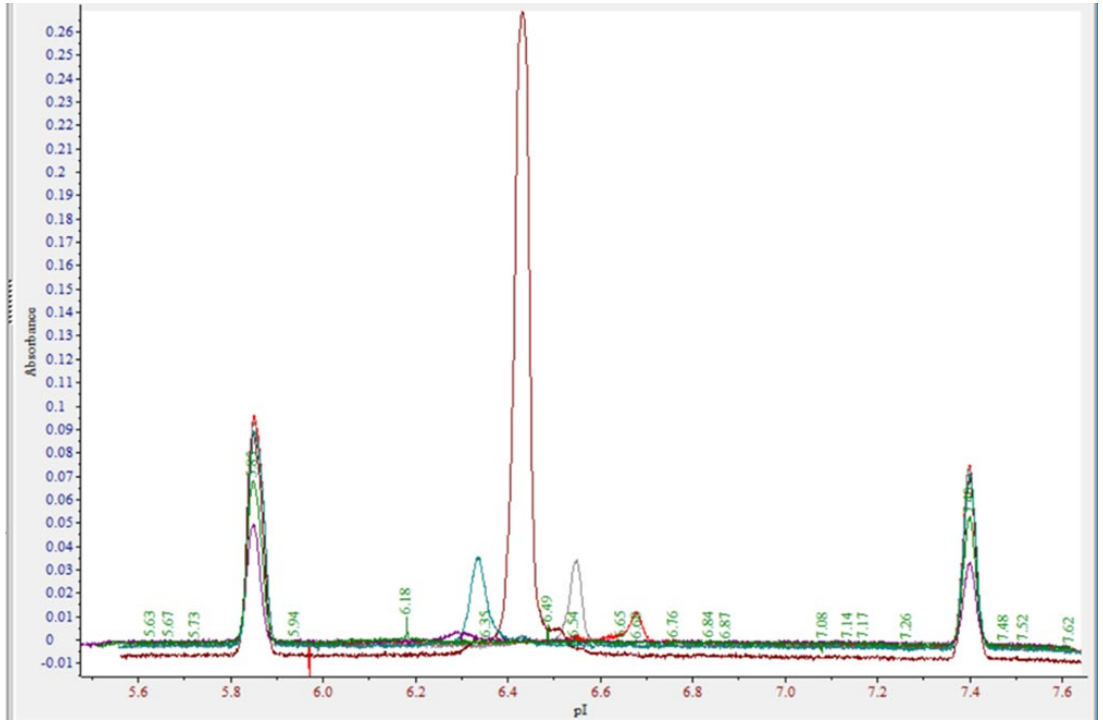
Principle of preparative iCIEF by AES

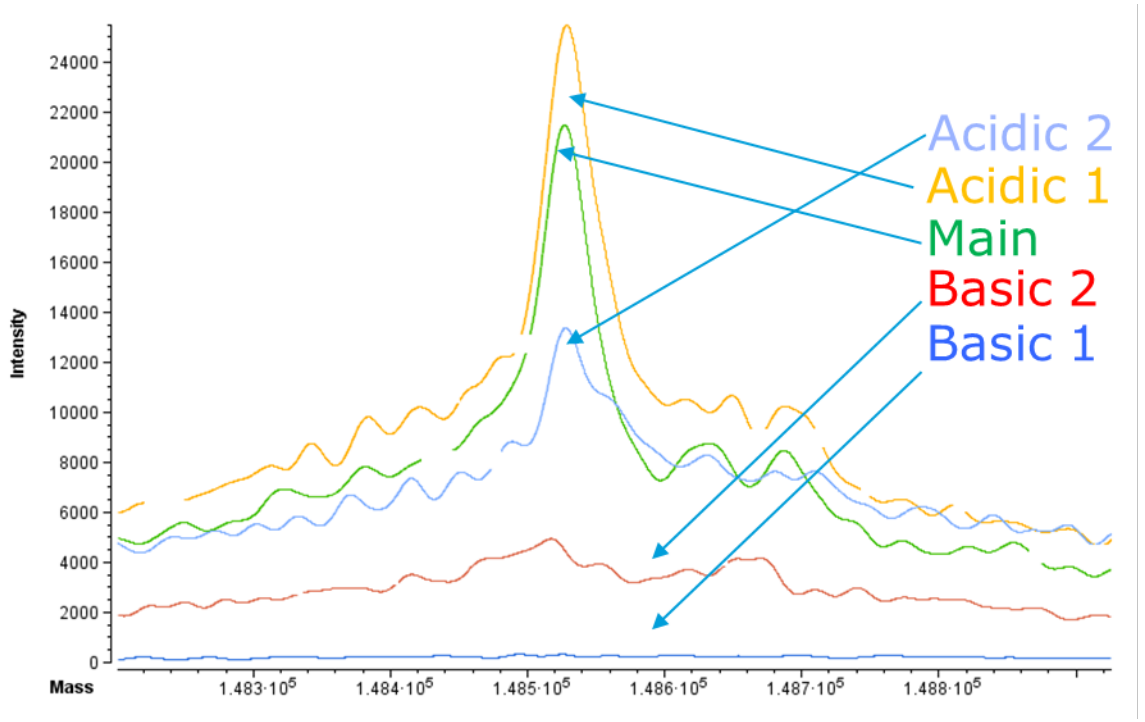


iCIEF – Fractionation



MS characterization of the charged isoforms





LC-MS setup: Bruker Maxis II ETD TOF, 1 x 150 mm id BEH C4 column

Data courtesy of Dr Carsten Sönksen, Novo Nordisk S/A

- Thank you for your interest and attention
- Hand-out of the presentation will be available.
- Submit your request at https://www.rozing.com