Markers of isoelectric point

an essential tool in capillary isoelectric focusing

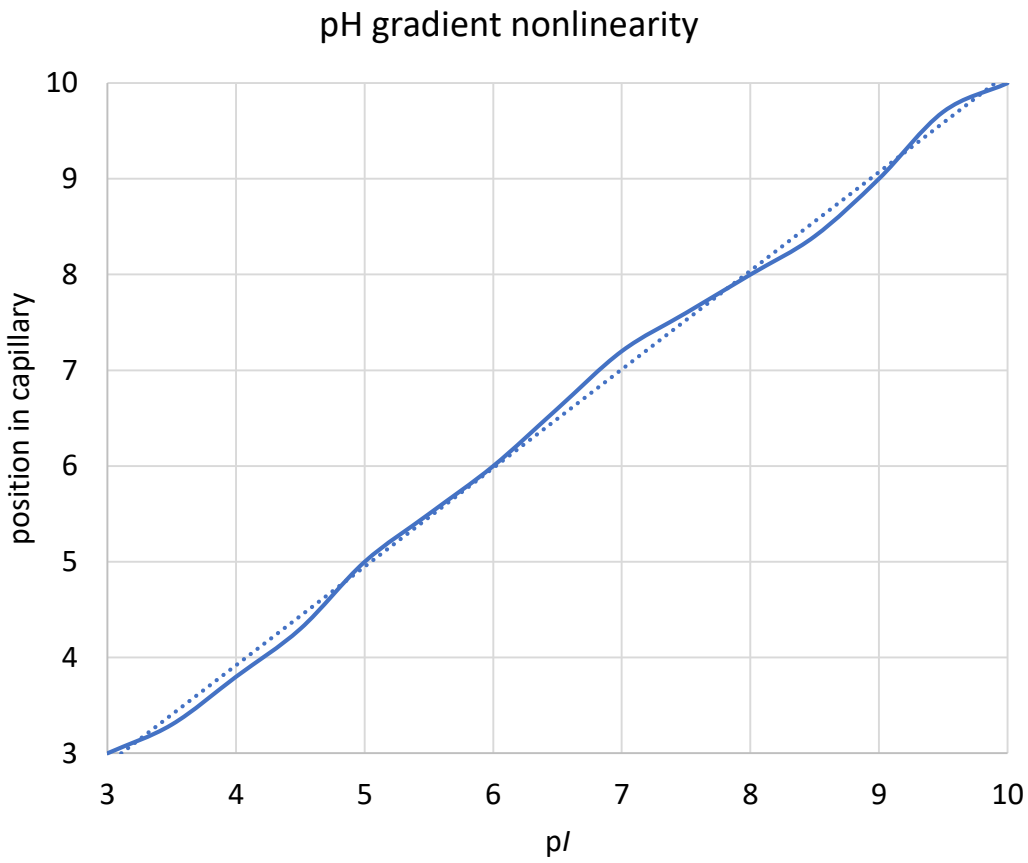
Filip Duša

Institute of Analytical Chemistry of the Czech Academy of Sciences

Capillary isoelectric focusing a powerful separation method

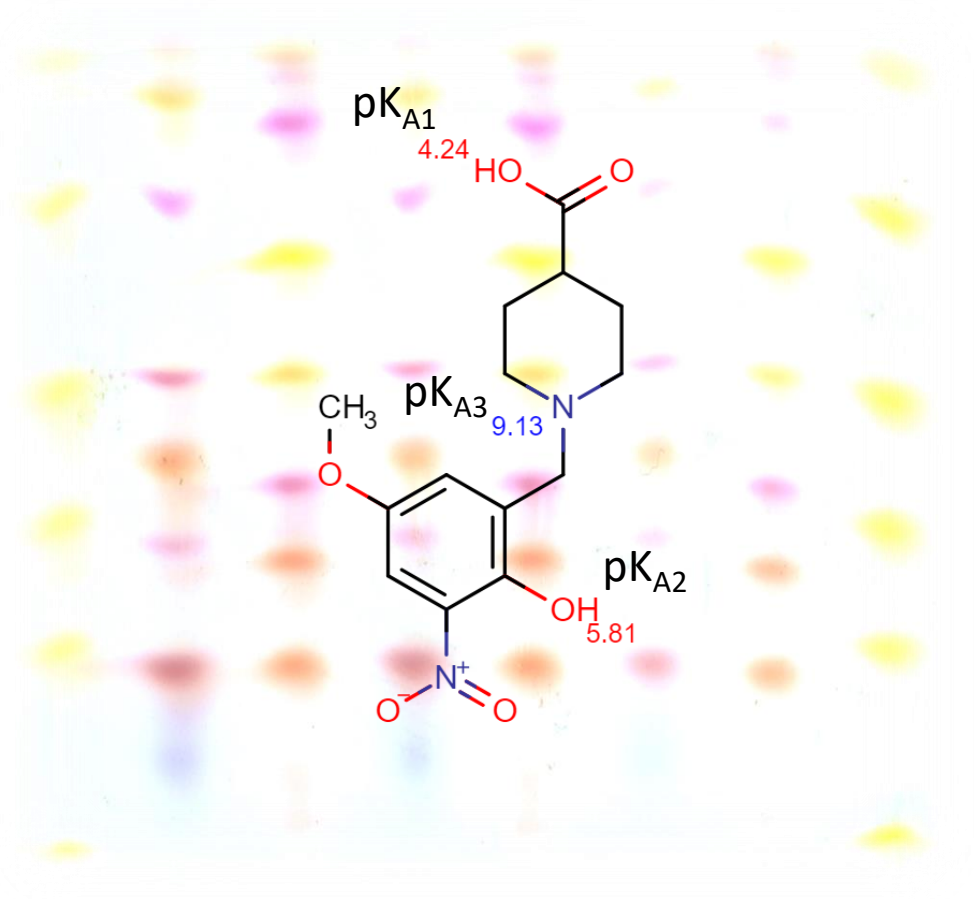
+ Resolution as low as 0.01 pH unit

- Repeatability issues
  - pH gradient formation – mixtures of hundreds of compounds (carrier ampholytes)
  - Surface quality/coating stability
  - Localized Joule heating → careful control of the total applied electric power
  - Two phases of the separation – focusing and mobilization
  - Influence of sample matrix (*e.g.*, salts, buffers)



Internal standards of isoelectric focusing – isoelectric point (p*I*) markers

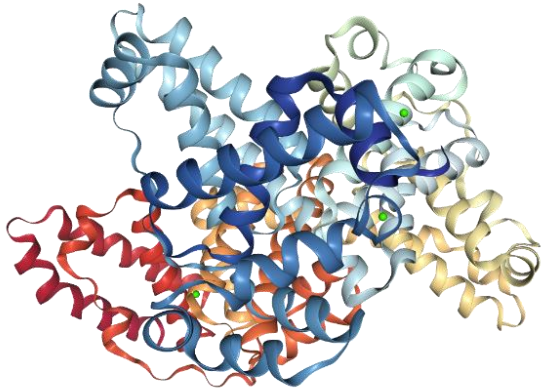
- Critical qualities of p*I* markers
  - Well-focusing ampholyte
    - $\Delta pK_A < 4$
  - Solubility at p*I*
  - Detectability at 280 nm
    - Absorption of carrier ampholytes below 280 nm
  - Observable at visual spectrum
  - Stability in solution



A brief note on development of pI markers

Proteins

- Naturally occurring
- Have to be extracted from matrices
- High heterogeneity → isoforms, posttranslational modifications
- Low stability against degradation

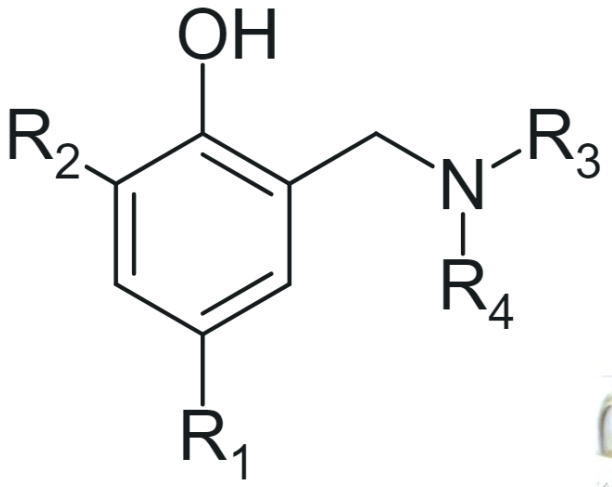


Peptides

- Based on tryptophan (absorption at 280 nm)
- Designed and synthesized on demand (Shimura et al., 2000 →)
- High purity
- Low heterogeneity
- Low stability at ambient temperature

Low-molecular-mass (LMM) p*I* markers

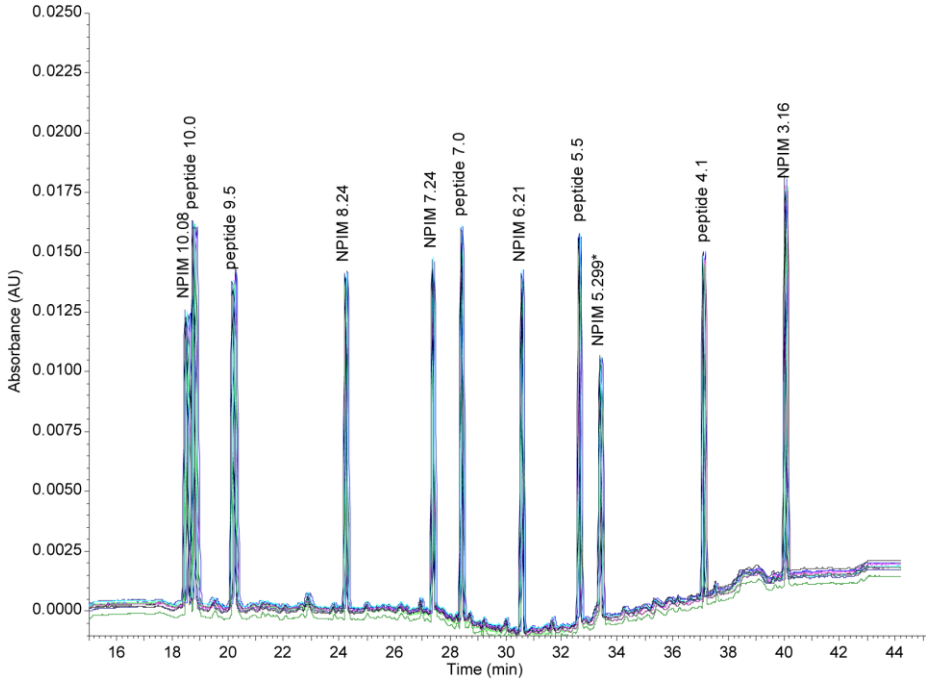
- Simple well-absorbing core, that can be easily modified by attaching functional groups
- Preferably one-step synthesis
- Fulfilling p*I* marker requirements
- First development and synthesis from 1994* and continues till present
- Universality, selectivity, sensitivity



*Šlais, K., Friedl, Z. J. Chromatogr. A 1994, 661, 249–256.

Universality
Nitrophenol based LMM pI markers (NPIM)

- Yellow color of the pI markers*
- λ_{MAX} = 400 – 500 nm → visual detection
- Low molecular mass → fast focusing
- High stability in powder and in solution
- 11 NPIMs calibrated using commercially available peptide pI markers
- MS detectable**

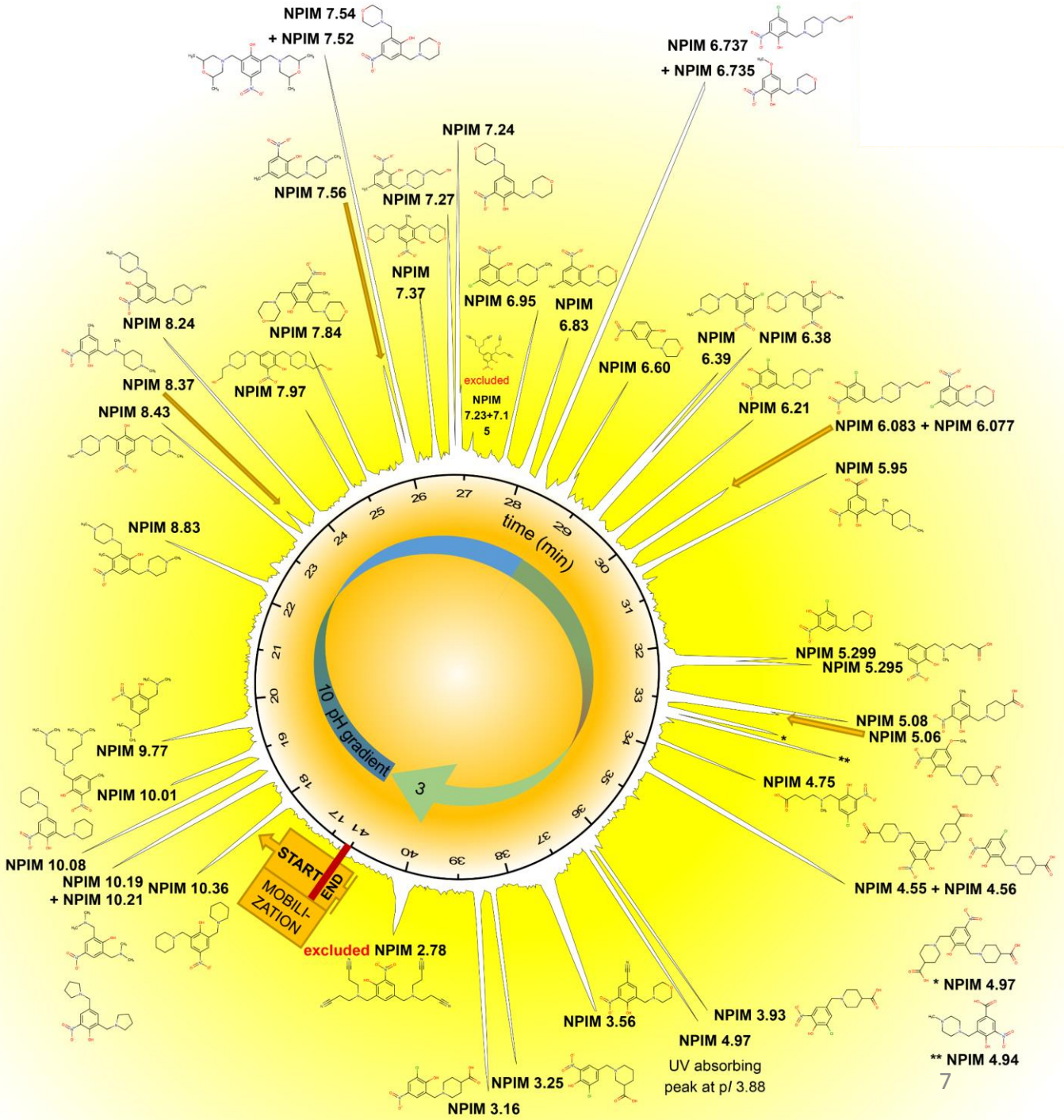


Method:
Sciex PACE MDQ Plus; 50 µm i.d. eCAP NEUTRAL capillary, l_{tot} = 30.2, l_{eff} = 20 cm; focusing 15 min at 25 kV; mobilization for 30 min at 30 kV; UV detection at 280 nm
Sample:
2.4M Urea, 80% V/V cIEF gel, 1.92% w/V Pharmalyte 3-10, 40mM L-Arginine, 1.6mM Iminodiacetic acid

*Duša, F., Moravcová, D., Šlais, K. Anal. Chim. Acta 2019, 1076, 144–153.
**Mazanec, K., Bobalová, J., Šlais, K. Anal. Bioanal. Chem. 2009, 393, 1769–78.

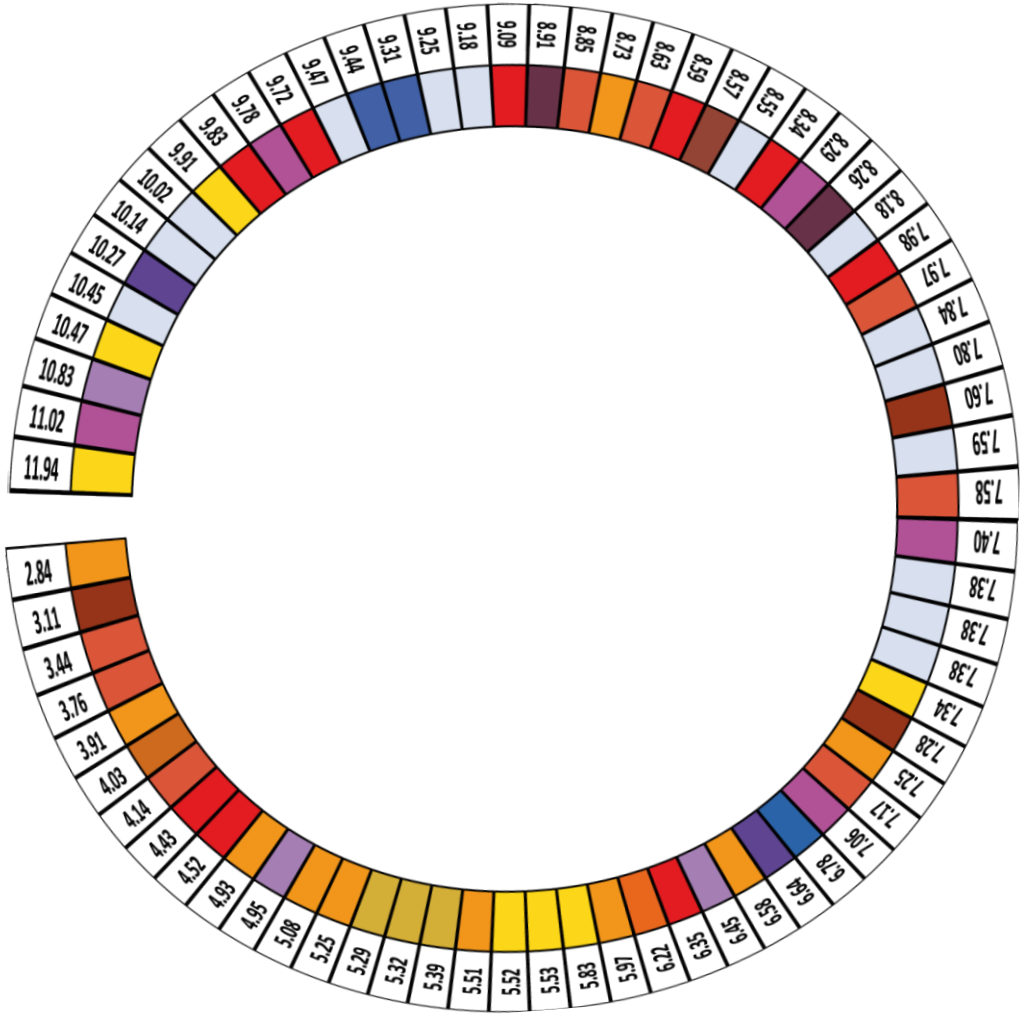
41 NPIMs developed and characterized

- 3.16 – 10.36 pH range covered
- pH coverage gaps identified
  - 9.77 – 8.43
  - 5.95 – 5.29
  - 4.55 – 3.93
- Hard to distinguish individual markers

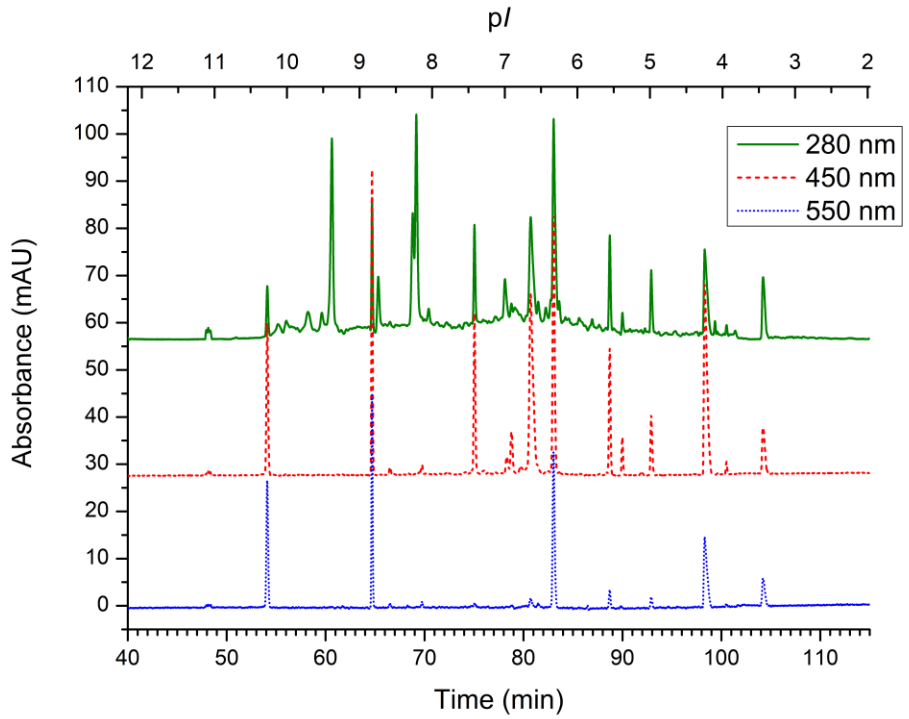


Selectivity
Colored p*I* markers (CPIM)

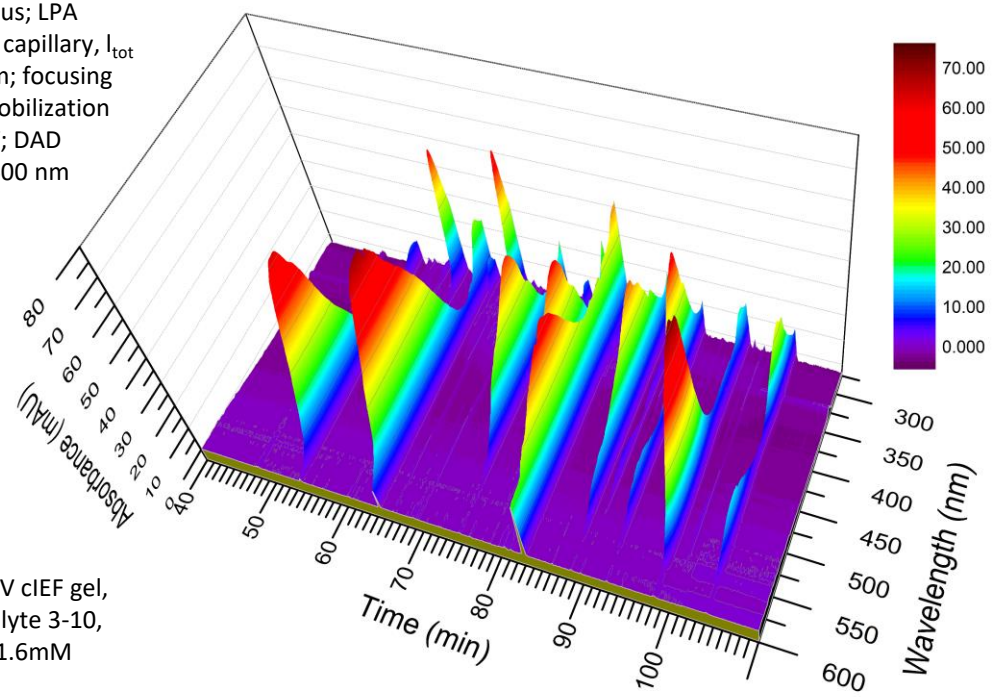
- Further UV-Vis absorbing cores added
  - Azo dyes based PIMs – 41 compounds
  - Sulfonephthalein/Phthalein based PIMs – 17 compounds
  - Phenol based PIMs (UV absorption only) – 14 compounds



Differently absorbing p*I* markers enable direct identification



Method:
Sciex PACE MDQ Plus; LPA coated 100 µm i.d. capillary, l_{tot} = 40 cm, l_{eff} = 30 cm; focusing 40 min at 20 kV; mobilization for 85 min at 25 kV; DAD detection at 275 -600 nm

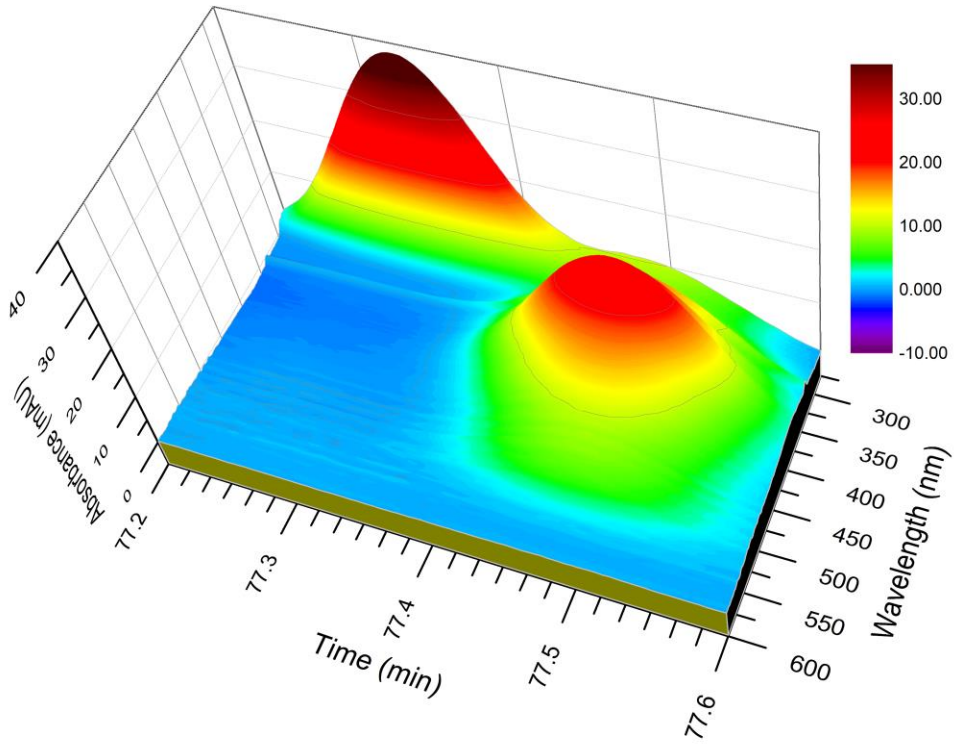


Sample:
2.4M Urea, 80% V/V cIEF gel, 1.92% w/V Pharmalyte 3-10, 40mM L-Arginine, 1.6mM Iminodiacetic acid

Duša, F., Moravcová, D., Šlais, K. *Anal. Chim. Acta* 2022, *1221*, 340035.

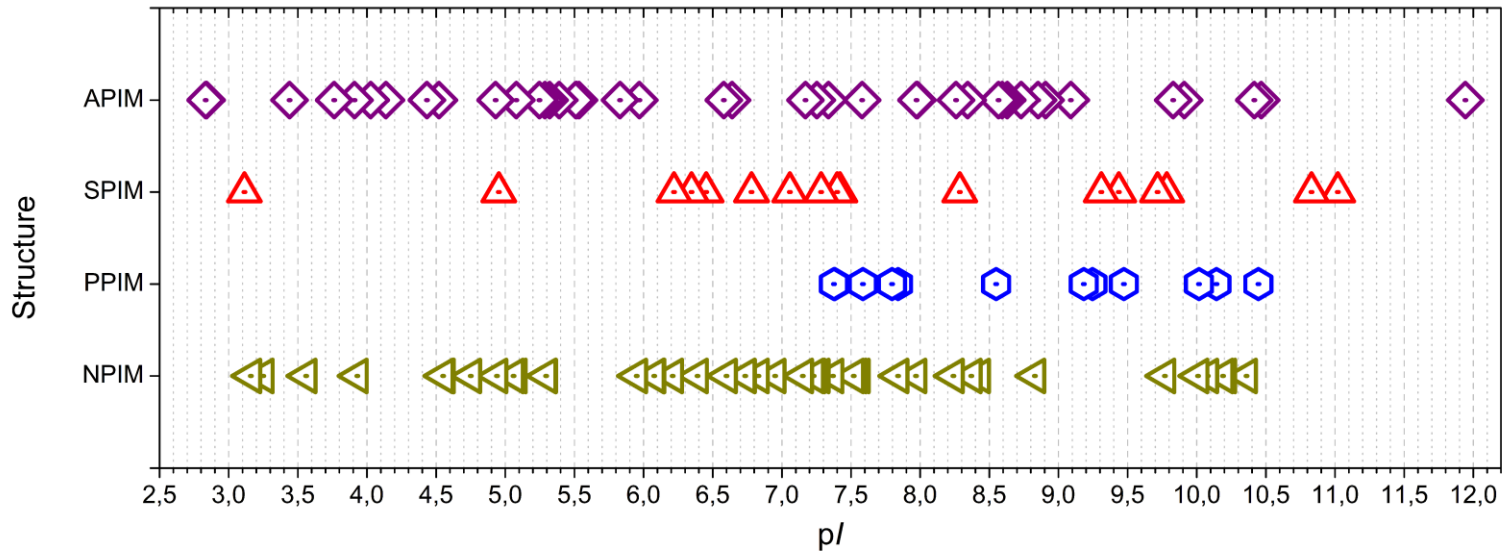
Coelution of PIM and analyte

- Example of coeluting compounds
  - peptide p*I* 5.50 (λ_{MAX} = 280 nm)
  - CPIM 5.52 (λ_{MAX} = 378 nm)
- Both compounds can be easily distinguished using 2 detection wavelengths
- fluctuations of pH gradient are diminished



pH gradient densely covered by the sum of PIMs

- Gaps present in the NPIM range were filled by CPIM
- Appropriate combination enabled to selectively determine each marker in the mixture
- 113 compounds available for tracing pH gradient



Sensitivity
Fluorescent p/ markers*

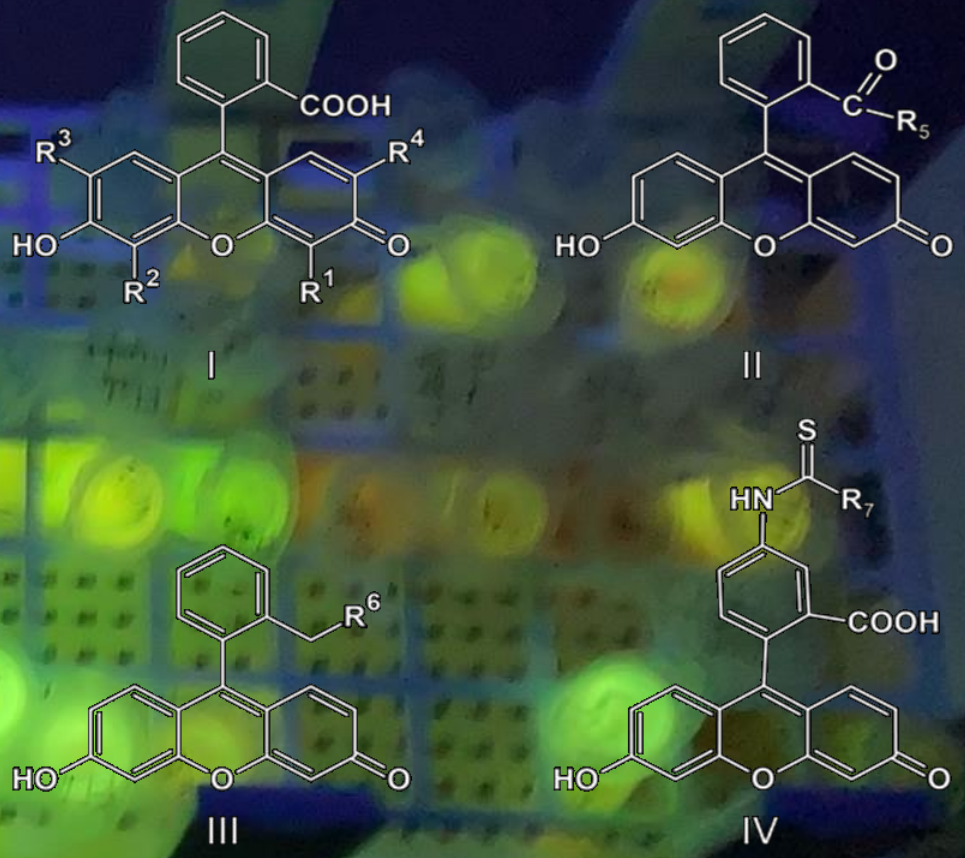
- Laser induced fluorescence
  - Detection limits down to pmol
- A new set of markers based on fluorescein core was developed
  - λ_{ex} ~ 488 nm, λ_{em} > 500 nm
- Types of synthesis:

  I – Mannich condensation

  II – Amid of amine

  III – red/ox of fluorescein

  IV – Fluorescein isothiocyanate



*Dadajová, P., Čmelík, R., Šlais, K., Duša, F., soon to be published data

Purity of the FPIMs

- Problematic synthesis
  - Contamination by byproducts
  - Low fluorescence of products
- cIEF-UV and cIEF-LIF analysis
  - 65 initial candidate structures
  - 19 FPIMs passed
    - purity (at least 50 % intensity of the main peak)
    - fluorescence intensity (0.5 peak area RFU)
- MS analysis was used to confirm the expected masses of the FPIMs passing the criteria

Purity table

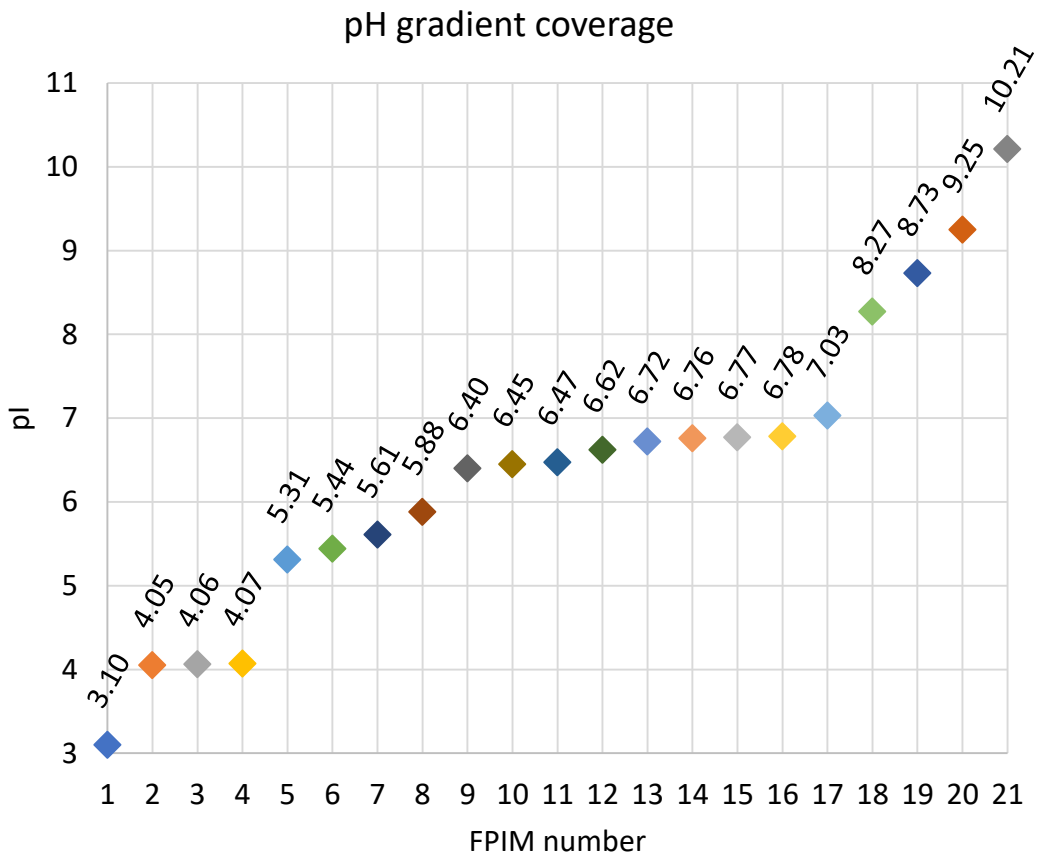
cIEF p/ main peak	cIEF čist.	cIEF p/ 2nd peak	2nd peak	RFU area/ng
6.33	100		0	1.6
4.68	100		0	0.9
6.33	100		0	0.3
2.09	100		0	0.1
6.64	100		0	0.1
6.63	100		0	0.1
6.57	100		0	0.0
5.55	97		0	331.5
6.43	97		0	12.4
6.33	97		0	3.9
6.79	96		0	75.4
6.79	95		0	9.4
6.94	95		0	4.7
6.07	95		0	2.5
6.81	92		0	8.1
6.79	92		0	1.5
6.53	90		0	2.5
6.38	89		0	0.5
6.73	88		0	9.6
3.99	87		0	14.4
6.34	86		0	1.4
6.33	85		0	1.6
6.38	85		0	0.5
4.91	85		0	0.3
6.49	82		0	75.3
5.78	82		0	4.8
6.65	82		0	0.1
6.07	81		0	1.3
6.64	80		20	116.5
5.44	80		0	2.5
6.4	80		0	0.4
2.31	76		0	0.2
9.53	74		**0**	0.2
2.21	73		0	0.2
5.03	71	5.03	0	0.3
4.76	67	5.70	27	2.2
6.56	67		0	1.8
6.29	67		0	0.8
6.79	66		0	0.9
3.03	63	3.34	23	1.3
6.47	61	5.36	31	4.0
9.51	58		**0**	0.2
6.53	56		0	3.0
8.32	55	6.63	36	1.1
5.05	55	5.10	19	0.4
3.85	50	4.12	47	1.4
7.68	50		0	0.1
8.87	49		0	0.1
6.58	48	5.39	52	0.8
8.35	47	6.70	34	1.1
4.78	47		0	0.1
4.84	45	4.91	40	0.7
10.3	45	9.6	0	0.1
3.79	43		0	1.4
9.02	40		0	0.3
10.3	40	9.6	0	0.1
8.2	35		0	0.1
5.29	33	6.46	46	4.2
10.3	30	9.6	0	0.1

Fluorescence intensity table

cIEF p/ main peak	cIEF čist.	cIEF p/ 2nd peak	2nd peak	RFU area/ng
5.55	97		0	331.5
6.64	80		20	116.5
6.79	96		0	75.4
6.49	82		0	75.3
3.99	87		0	14.4
6.43	97		0	12.4
6.73	88		0	9.6
6.79	95		0	9.4
6.81	92		0	8.1
4.93	10	3.87	90	5.4
5.78	82		0	4.8
6.94	95		0	4.7
5.29	33	6.46	46	4.2
6.47	64	5.36	31	4.0
6.33	97		0	3.9
6.53	56		0	3.0
8.3	6		0	2.7
6.07	95		0	2.5
5.44	80		0	2.5
6.53	90		0	2.5
4.76	67	5.70	27	2.2
6.56	67		0	1.8
6.33	100		0	1.6
6.33	85		0	1.6
6.79	92		0	1.5
6.34	86		0	1.4
3.79	43		0	1.4
3.85	50	4.12	47	1.4
6.07	81		0	1.3
3.03	63	3.34	23	1.3
8.32	55	6.63	36	1.1
8.35	47	6.70	34	1.1
6.07	27	6.07	50	1.0
6.79	66		0	0.9
4.68	100		0	0.9
6.58	48	5.39	52	0.8
6.29	67		0	0.8
4.84	45	4.91	40	0.7
6.38	89		0	0.5
6.38	85		0	0.5
6.4	80		0	0.4
5.05	55	5.10	19	0.4
6.63	20	3.72	73	0.4
5.03	71	5.03	0	0.3
4.91	85		0	0.3
9.02	40		0	0.3
6.33	100		0	0.3
9.53	74		**0**	0.2
2.31	76		0	0.2
2.21	73		0	0.2
9.51	58		**0**	0.2
10.3	45	9.6	0	0.1
2.09	100		0	0.1
6.64	100		0	0.1
8.2	35		0	0.1
10.3	40	9.6	0	0.1
7.68	50		0	0.1
8.87	49		0	0.1
6.65	82		0	0.1
4.78	47		0	0.1
6.63	100		0	0.1
10.3	30	9.6	0	0.1
8	17	6.83	57	0.05
6.57	100		0	0.0

FPIMs
Coverage of pH gradient

- Most of the compounds had p*I*s at neutral and acidic region
- Only a few FPIMs available for marking the basic pH
- None of the FPIM above the p*I* 8.73 passed the 50 % purity criterium
  - The criterium lowered to 40 % to add two more basic FPIMs

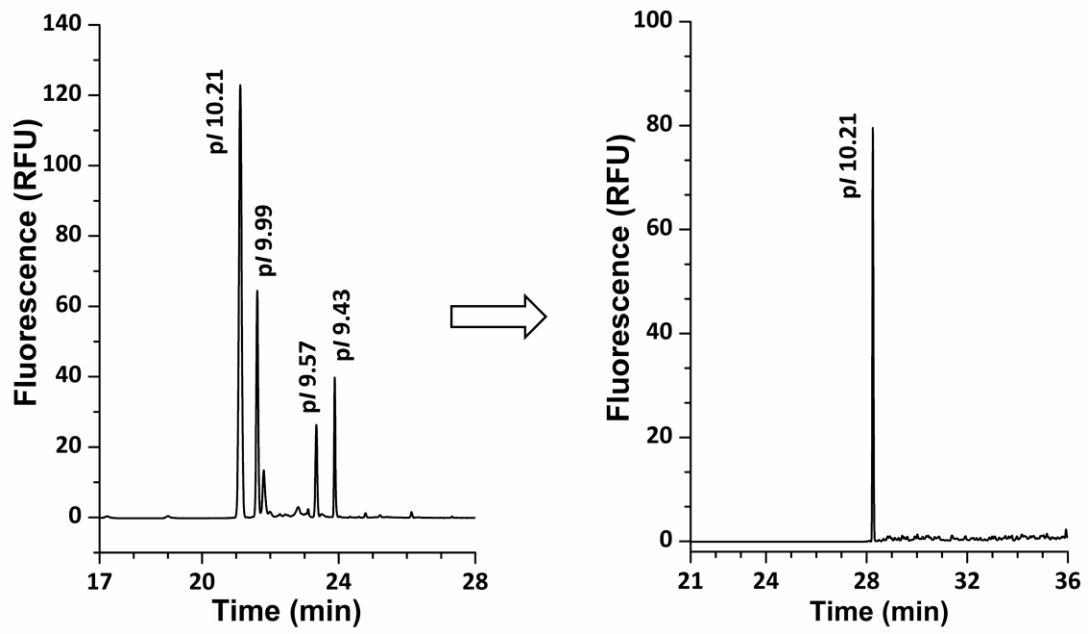


cIEF purification/fractionation of FPIM 10.21

- The final product of the FPIM synthesis contained impurities at pH range 9.4 – 10.2

Purification:

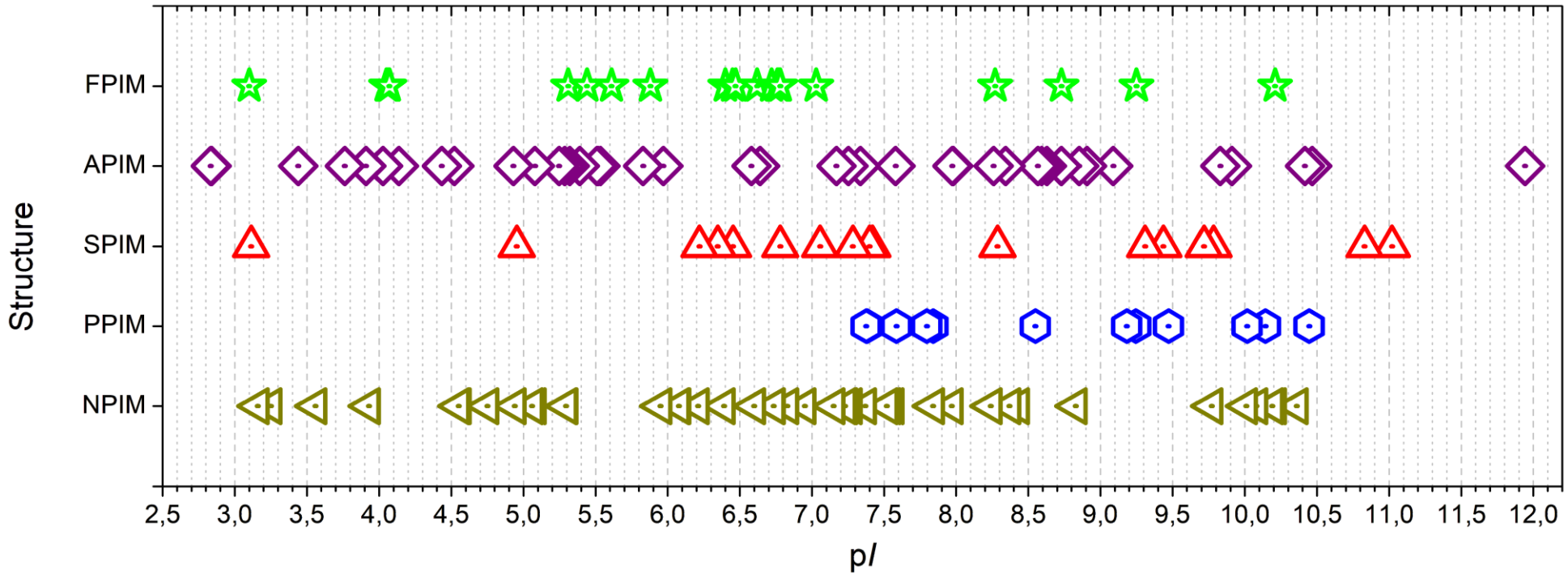
- 75 µm i.d. capillary
- Lysine (p*I* 9.7) spacer to separate the impurities in cIEF
- 9 fractions collected to chemical mobilizer solution (HAc) and vacuum-evaporated



Method:
Sciex PACE MDQ Plus; LPA coated 75 µm i.d. capillary, l_{tot} = 30 cm, l_{eff} = 20 cm; focusing 17 min at 20 kV; mobilization 11 min at 20 kV; fractionation 9 times 2 min at 20 kV; detection: argon-ion laser 488 nm, notch filter 488 nm / bandpass filter 520/20 nm.
Sample:
1.92 M Urea, 64 % V/V cIEF gel, 0,64 % w/V Pharmalyte 3-10, 120 mM L-Arginine, 1.6 mM Iminodiacetic acid, 15 ng mL^{-1} FPIM 10.21

Universality, Selectivity, and Sensitivity
LMM p*I* markers

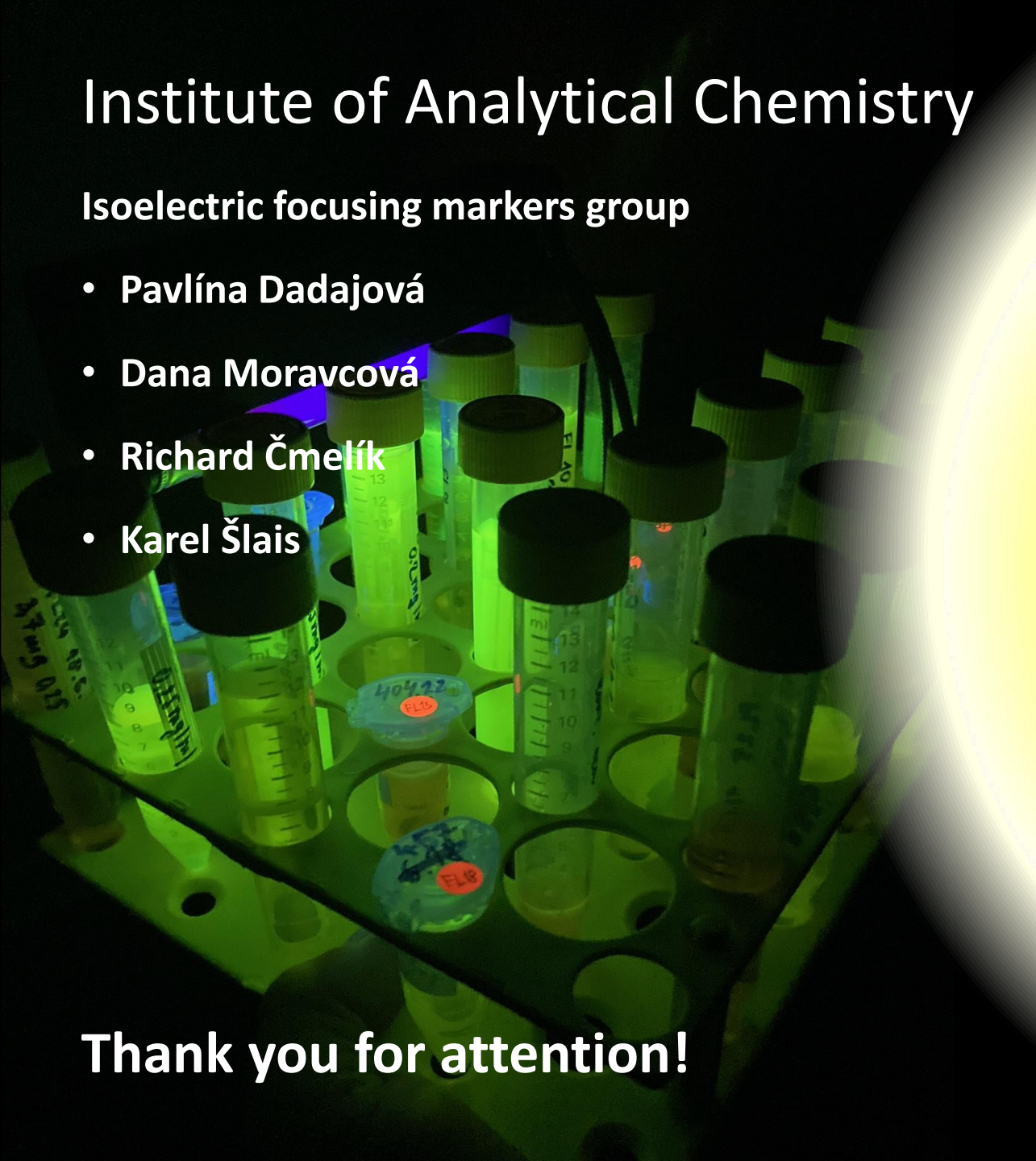
Total of 134 compounds for tracing pH gradient

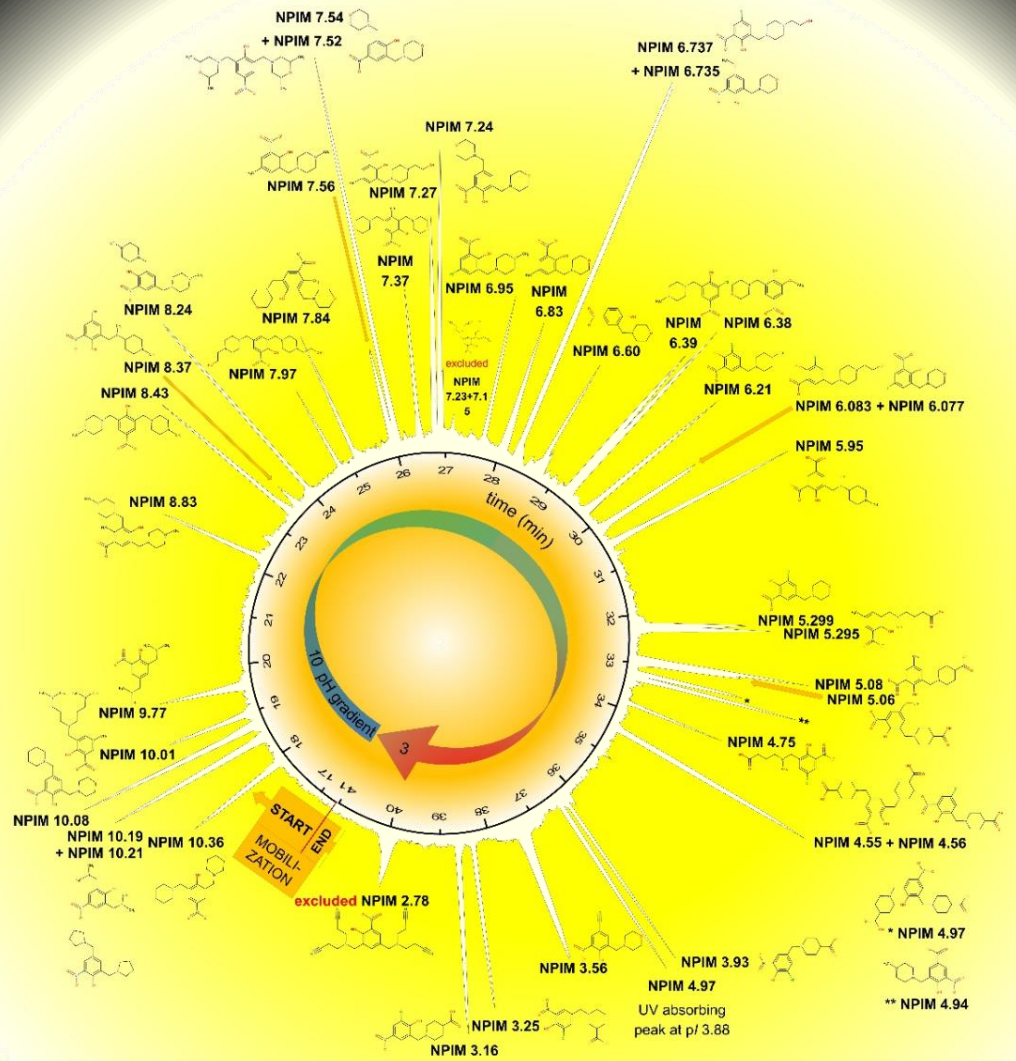


Institute of Analytical Chemistry

Isoelectric focusing markers group

- **Pavlína Dadajová**
- **Dana Moravcová**
- **Richard Čmelík**
- **Karel Šlais**





Thank you for attention!