Fluorescent compounds as pH tracing standards for capillary isoelectric focusing analysis of labeled proteins and their glycoforms using laser-induced fluorescence detection

PBA 2024 34th International Symposium on Pharmaceutical and Biomedical Analysis September 9-12, Geneva / Switzerland PS01-23

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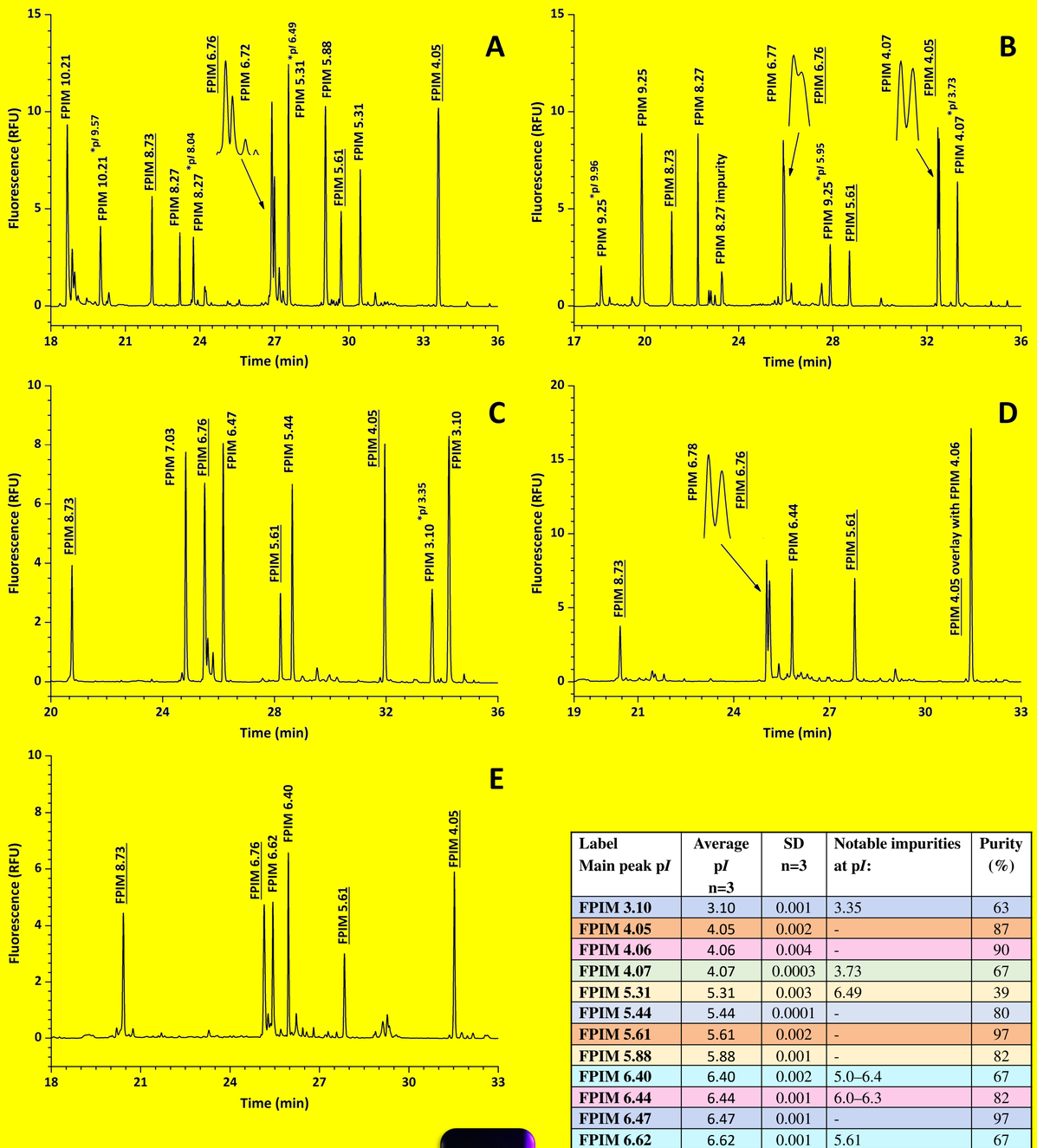
Introduction

Capillary isoelectric focusing (cIEF) is a powerful analytical method for the separation of proteins and their post-translational modifications. However, it suffers from lower limits of detection of the analyzed compounds when the absorbance is measured at the standard UV wavelength of 280 nm. Laser-induced fluorescence detection (LIF) is able to lower the detection limit by several orders of magnitude by using suitable fluorescent labels or fluorescent analytes. The trancing of a pH gradient by fluorescent compounds in cIEF-LIF is essential for method optimization and isoelectric point (p*I*) calibration of the compounds analyzed. We present a set of low-molecular-mass fluorescein-based compounds covering the pH range from 3.10 to 10.21. Thanks to the fluorescein core, the p*I* standards (markers) have an excitation range suitable for the most common 488 nm laser, with fluorescence emission usually collected above 500 nm.

Results

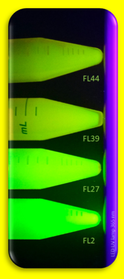
The novel fluorescent compounds were analyzed in regard to their purity after synthesis, their fluorescence properties, and focusing ability. Criteria were established and candidate compounds below the limits were removed from the group. After thorough analysis, a set of 19 fluorescent markers was selected as suitable for marking the pH gradient. In addition, due to the significant lack of fluorescent markers at basic pH two more candidates were added (first surpassing a lowered purity limit, the second being purified by cIEF fractionation).

cIEF-LIF calibration of the selected FPIMs



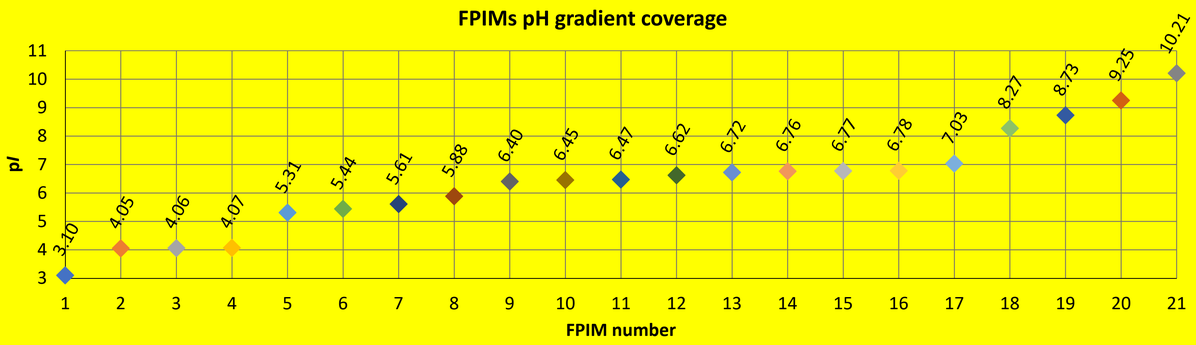
Four selected FPIMs were first calibrated using our yellow p*I* markers [2] and cIEF-UV method (FPIM standards, colored orange in table).
The rest of FPIMs was divided into five sets (A, B, C, D, and E) to prevent overlap and calibrated using the FPIM standards (each set colored individually in the table.

Impurities from synthesis on electropherograms are marked with asterix *



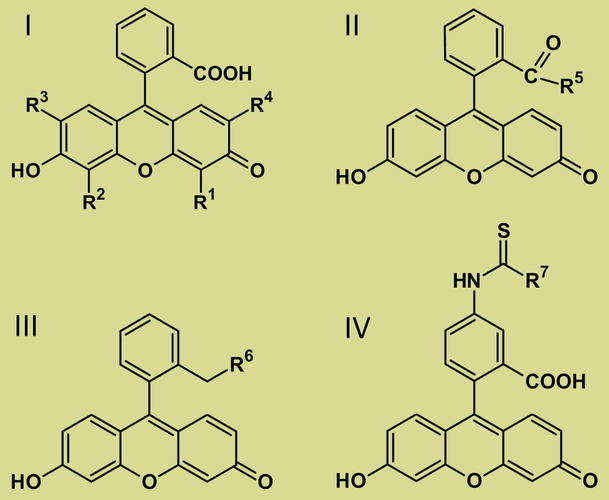
Label Main peak p*I*	Average p*I* n=3	SD n=3	Notable impurities at p*I*:	Purity (%)
FPIM 3.10	3.10	0.001	3.35	63
FPIM 4.05	4.05	0.002	-	87
FPIM 4.06	4.06	0.004	-	90
FPIM 4.07	4.07	0.0003	3.73	67
FPIM 5.31	5.31	0.003	6.49	39
FPIM 5.44	5.44	0.0001	-	80
FPIM 5.61	5.61	0.002	-	97
FPIM 5.88	5.88	0.001	-	82
FPIM 6.40	6.40	0.002	5.0–6.4	67
FPIM 6.44	6.44	0.001	6.0–6.3	82
FPIM 6.47	6.47	0.001	-	97
FPIM 6.62	6.62	0.001	5.61	67
FPIM 6.72	6.72	0.003	-	80
FPIM 6.76	6.76	0.001	-	92
FPIM 6.77	6.77	0.001	-	92
FPIM 6.78	6.78	0.0004	-	95
FPIM 7.03	7.03	0.001	-	95
FPIM 8.27	8.27	0.001	7.8–8.3	60
FPIM 8.73	8.73	0.002	9.85	74
FPIM 9.25	9.25	0.001	6.0–6.1, 9.2–10.0	40
FPIM 10.21	10.21	0.008	-	92

FPIMs pH gradient coverage



p*I* marker synthesis

To obtain the described set of fluorescent p*I* markers (**FPIMs**), four different synthetic routes were used to modify the fluorescein core.
FPIMs of the general structure **I** represent the majority of the presented markers. They were prepared by Mannich reaction of fluorescein or dichlorofluorescein with formaldehyde and appropriate amine in aqueous environment. The reaction was carried out at room temperature from 20 h to several days, depending on the product. Then the pH of the reaction mixture was adjusted with diluted hydrochloric acid to the desired p*I*. The precipitated product was collected and recrystallized from aqueous ethanol and dried.
Synthesis of the FPIMs with general structure **II** was described previously [1].
The general structure **III** pI marker was prepared by treatment of the corresponding amide **II** with mild reduction agent $NaBH_4/I_2$. Finally, the obtained dihydrofluorescein compound was oxidized using H_2O_2 to provide fluorescent product again.
Fluorescein isothiocyanate (FITC) was used for preparation of the pI markers of the general structure **IV**. The solution of FITC and triethylamine in DMF was treated with appropriate amine overnight in a dark place at ambient temperature.



cIEF method

- Instrument: Sciex P/ACE™ MDQ Plus DAD (280 nm) and LIF (excitation 488 nm/emission 520±10 nm), neutral linear polyacrylamide capillary 30.2 cm long (20 cm effective length), tempered to 20 °C
- Sample: 2.4 M urea, 80% V/V cIEF gel, 1.92% w/V background ampholytes (Pharmalyte 3-10), 60 mM L-arginine and 1.6 mM iminodiacetic acid
- Separation conditions: Two step cIEF – focusing 200 mM H_3PO_4 and 300 mM NaOH (30 kV, 15 min), chemical mobilization with 350 mM CH_3COOH (25 kV).

Image by AB Sciex LLC, https://sciex.com/products/capillary-electrophoresis/p-ace-mdq-plus

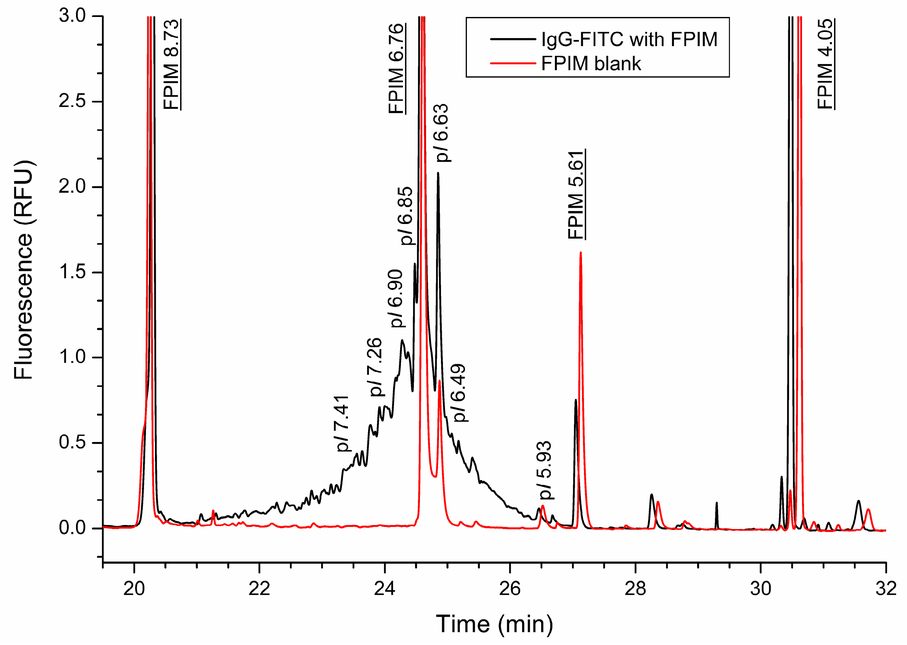
cIEF-LIF applications

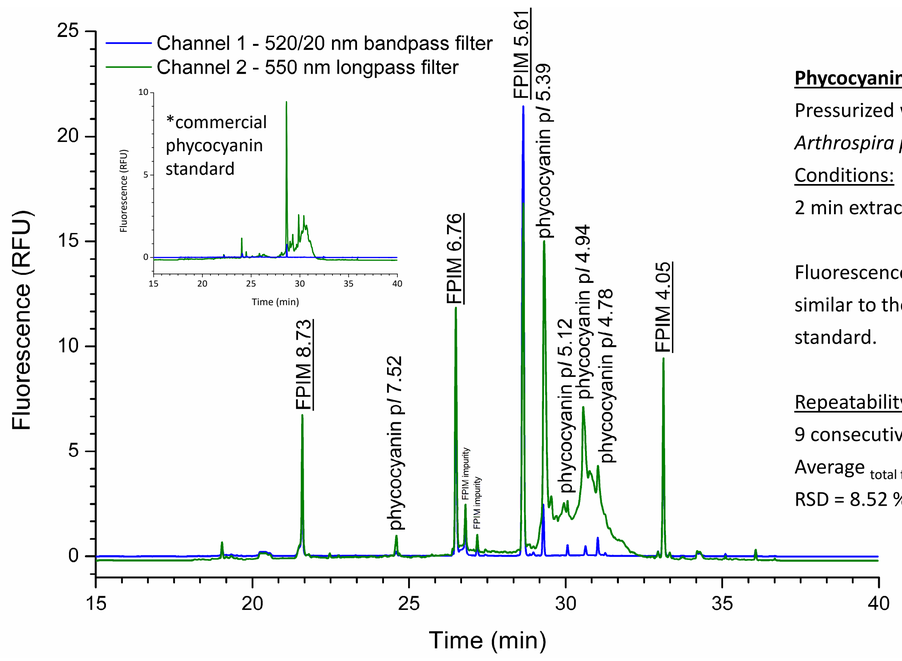
IgG-FITC

FITC labeled human IgG standard (70 nmol·l^{-1}) was used as an illustrative application. Labeled variants of IgG and its glycoforms stretched (focused) almost over the 2 pH units range.

For the most precise sample p*I* determination of IgG-FITC variants:

- Two FPIMs as closely bordering the IgG-FITC variants as possible
- One (optional) FPIM „inside“ the IgG-FITC focused zone





Phycocyanin extract

Pressurized water extraction from algae *Arthrospira platensis* (spirulina) powder

Conditions:

2 min extraction at 40 °C and 15 MPa

Fluorescence profile of the extract was very similar to the commercially obtained protein standard.

Repeatability:

9 consecutive extractions

Average $_{\text{total fluorescence area}}$ = 48.6·10^6 RFU

RSD = 8.52 %

Conclusions

Comprehensive set of 21 low-molecular-mass FPIMs enables detailed tracing of the pH gradient in cIEF-LIF applications.
Utilizing such set of compounds enabled precise calibration of the pIs of very low amount of injected proteins. This approach was demonstrated using fluorescently labeled immunoglobulin glycoforms and naturally fluorescent protein phycocyanin.

Acknowledgments

This work was supported by the Grant Agency of the Czech Republic [22-00236S] and the institutional research plan [RVO:68081715].

References

[1] https://doi.org/10.1002/1522-2683(200206)23:11<1682::AID-ELPS1682>3.0.CO;2-Q

[2] https://doi.org/10.1016/j.aca.2019.05.010

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