Fluorescein-based isoelectric point markers as a tool for tracking of pH gradient in highly sensitive isoelectric focusing analysis with laser induced fluorescence detection

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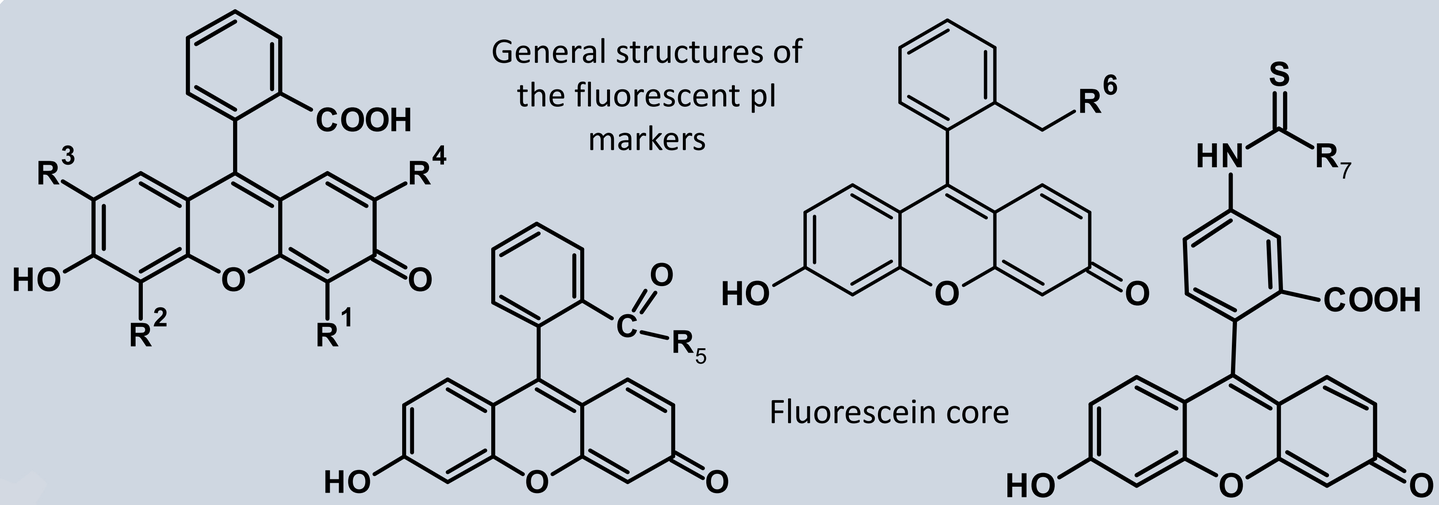
Isoelectric point (p*I*) markers are crucial internal standards used in isoelectric focusing (IEF) methods for tracking the pH gradient. Our laboratory has already introduced colored (UV and visible region absorbing) low-molecular-mass (LMM) p*I* markers for capillary isoelectric focusing (cIEF) as an alternative to the commonly used peptide and protein p*I* markers. The cIEF instrumentation can be coupled with on-line laser induced fluorescence (LIF) detection, which significantly increases detection sensitivity. However, there was considerable lack of suitable p*I* markers for this type of application. Consequently, our laboratory devised fluorescein-based low-molecular p*I* markers, initially covering narrow pH range (from 5.4 to 6.6). By adding various functional groups to the fluorescein core. Of all the synthetized and tested candidate structures 21 markers were identified as suitable for the cIEF-LIF method.

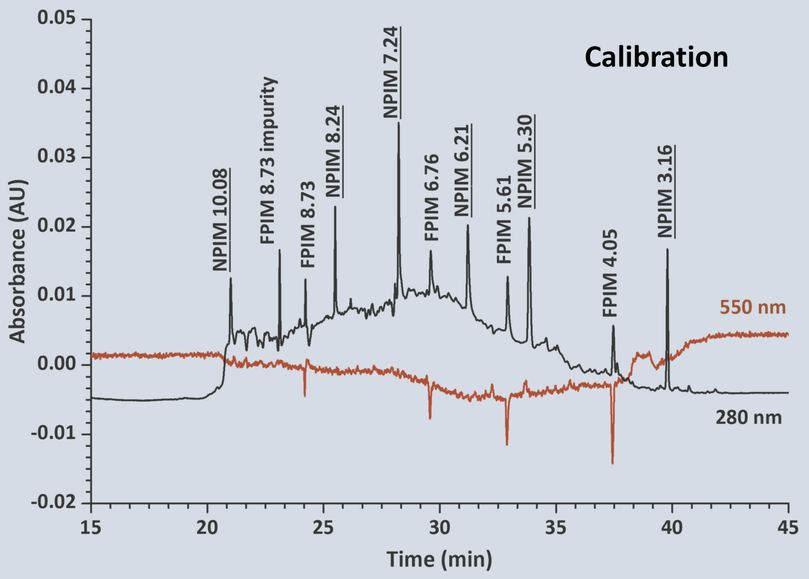


Label Main peak pI	SD n=3	Notable impurities at pI:	Purity (%)
FPIM 3.10	0.001	3.35	63
FPIM 4.05*	0.002	-	87
FPIM 4.06	0.004	-	90
FPIM 4.07	0.0003	3.73	67
FPIM 5.31	0.003	6.47	39
FPIM 5.44	0.0001	-	80
FPIM 5.61*	0.002	-	97
FPIM 5.88	0.001	-	82
FPIM 6.40	0.002	5.0–6.4	67
FPIM 6.45	0.002	6.0–6.3	82
FPIM 6.47	0.001	-	97
FPIM 6.62	0.001	5.61	67
FPIM 6.72	0.003	-	80
FPIM 6.76*	0.001	-	92
FPIM 6.77	0.001	-	92
FPIM 6.78	0.0004	-	95
FPIM 7.03	0.001	-	95
FPIM 8.27	0.001	7.8–8.3	60
FPIM 8.73*	0.002	9.85	74
FPIM 9.25	0.001	6.0–6.1, 9.2–10.0	40
FPIM 10.21	0.008	9.4–10.2	45

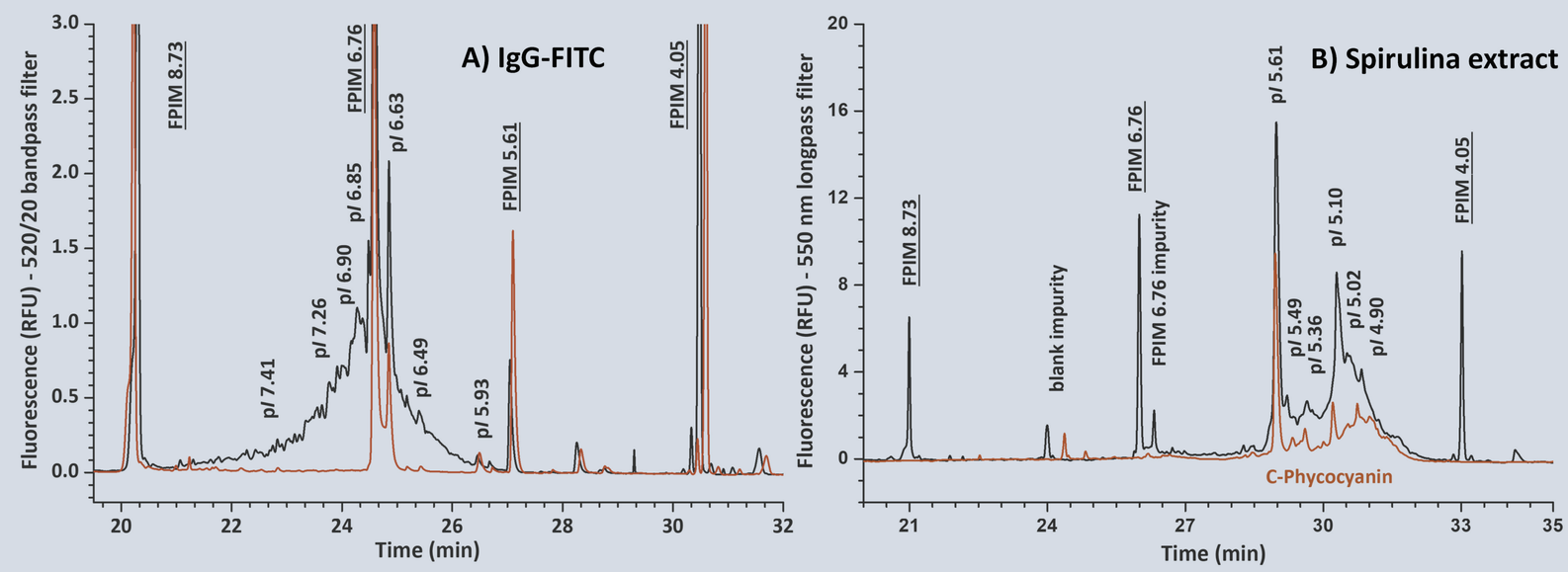
Instrument: Sciex P/ACE™ MDQ Plus, DAD (280 nm) and LIF (ex 488/em bandpass 520/20 nm and longpass 550 nm), neutral LPA capillary 30.2/20 cm, cooling at 20°C

Separation conditions: Two step cIEF – focusing 200 mM H3PO4 and 300 mM NaOH (30 kV, 15 min), chemical mobilization with 350 mM CH3COOH (25kV).

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



➤ Purity assessment and calibration of four selected fluorescent markers (marked by * in the table) in UV 280 nm with our yellow nitrophenol p*I* markers. Calibration of the remaining markers was with LIF detection.



➤ Application of the markers in p*I* determination and charcterization of **A)** commercialy labeled antibody - IgG-FITC (λ_{max} ~ 512 nm), where multiple labeled isoforms of the antibody can be observed and **B)** extract from *Arthrospira platensis* (Spirulina) with comparison to a C-Phycocyanin standard (λmax ~ 640 nm), which is at its p*I* present in a monomer-trimer system.

CONCULSION

We present 21 new characterized fluorescent ampholytic structures for tracking of the pH gradient. Their p*I* values have been determined with precision of one hundred of pH unit using a linear calibration based on four selected markers. The selected structures have been proved to be p*I* markers featuring good focusing abilities, manifested as well-defined narrow peaks that can be easily applied in IEF methods with fluorescence detection and cover the range from 3.10 to 10.21, which greatly expands the selection of fluorescent p*I* markers.

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